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We are investigating the role of the AP-1 transcription complex in regulating the growth of normal and malignant breast cells. We have determined the levels of cJun and cFos protein expression and AP-1 transcriptional activity in normal HMECs, immortalized HMECs, oncogene-transformed HMECs, and breast cancer cell lines, and have determined the relative sensitivity of these different cells to a specific AP-1 inhibitor.

These studies demonstrated that there is a progressive decline in basal AP-1 activity as human mammary epithelial cells progress through the carcinogenesis pathway (normal HMEC->immortal HMEC->oncogene-transformed HMEC->breast cancer cell). Stimulation of AP-1 activity by EGF was observed in immortal, oncogene-transformed HMEC, and breast cancer cells but not normal HMECs. Normal HMECs, which had the highest AP-1 activity, were most sensitive to AP-1 inhibition, immortal HMEC, which have an intermediate AP-1 activity, were moderately sensitive, and breast cancer cells, having the lowest AP-1 activity, were relatively resistant to AP-1 blockade.

This data suggests that as human mammary epithelial cells progress towards malignancy they become less dependent on AP-1 transcriptional activity for proliferation. In ongoing studies we are now investigating whether the transformed phenotype of human breast cells depends on AP-1 activity. These studies will define the role of AP-1 in regulating growth and transformation of human breast cells.

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Powel Brown 10/20/97

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INTRODUCTION

Breast cancer is the most common malignancy in women, and the leading cause of death for women between the ages of 40 and 55 in this country (1). Even with aggressive mammographic screening, adjuvant chemotherapy, and intensive therapy for existing cancer, many of the women who develop breast cancer will die from it. Thus, more effective prevention strategies and treatments are urgently needed.

Unfortunately, little is known about the specific molecular events which cause the progressive transformation of human breast epithelial cells to malignant breast cancer. Studies of model systems of cancer have revealed that multiple steps are involved in carcinogenesis, including tumor "initiation" and "promotion" events (2). Mutations and deletions within tumor suppressor genes may represent the molecular equivalent of breast cancer "initiation" events (3,4). However, the molecular mechanism of breast tumor "promotion" is poorly defined. In model systems (5), classic tumor promoters induce the proliferation of initiated cells, leading to the progressive outgrowth of fully malignant cells. Such tumor promoters typically activate signal transduction pathways to stimulate cellular proliferation. In human breast cells, the overproduction of growth factors, or aberrant stimulation of growth factor receptors, may be responsible for the promotional phase of breast carcinogenesis. Growth factors important for mammary epithelial cells, such as estrogen, EGF, TGF-α, and the IGFs, may all represent tumor promoters of human breast cancer. Thus, drugs which inhibit the ability of estrogen to activate the estrogen receptor (tamoxifen and other antiestrogens) are used to treat breast cancer, and other drugs which block growth factor receptors, such as antibodies specific for the epidermal growth factor receptor and the Her2/neu receptor, have been shown to inhibit breast cancer cell proliferation (9-11) and are now being tested in clinical trials for the treatment of breast cancer. However, inhibition of individual signal transduction pathways may ultimately be ineffective, since breast cell proliferation can be stimulated by multiple different signal transduction pathways. It may be more effective to inhibit signal transduction at a more distal point in the cascade, where multiple mitogenic signals converge. Since transcription factors, the nuclear proteins which control DNA transcription and gene expression, are the most distal components of these converging mitogenic signal transduction pathways, they may be ideal targets for new therapeutic agents.

A key family of transcription factors transducing multiple mitogenic signals is the AP-1 family. These transcription factors are complexes of DNA-binding proteins made up of dimers of Jun and Fos proteins which bind DNA at specific AP-1 sites and regulate the transcription of AP-1-dependent genes. AP-1 transcription factors are expressed in most cell types, and are activated by specific kinases, such as the mitogen-activated and stress-activated kinases, which are themselves activated by diverse signals such as growth factor stimulation, exposure to UV light, oxidative stress, tumor promoters such as TPA, or oncogene overexpression or activation (reviewed in 6). Thus, AP-1 is a central component of many signal transduction pathways in a variety of cell types.

Previous studies showed that the AP-1 transcription factor family is critical for growth factor induced proliferation of fibroblasts (12,13). In addition, we (14,15) and others (16) have shown that AP-1 is also critical for oncogene-induced transformation of fibroblasts. Specifically, we have demonstrated that AP-1 is critical for the cotransformation of primary rat embryo cells by *ras+jun*, *ras+fos*, or *ras+SV40 T antigen* (14), while others have shown that AP-1 is critical for the transformation of NIH3T3 cells by single oncogenes such as *ras*, *raf*, *abl*, and *mos* (16). Thus, AP-1 is a central regulator of transformation as well as mitogenic signaling.

While the role of AP-1 has been extensively studied in fibroblasts, relatively few studies of the function of AP-1 have been performed in epithelial cells, and thus the exact role of this transcription factor family in controlling the proliferation and transformation of epithelial cells is not known. Previous studies from our lab and others have demonstrated that the Jun and Fos family members are expressed in human breast cancer cells, and are activated by a variety of important growth factors for these cells, such as EGF, TGFα, and the IGFs. Recent studies from other laboratories have also suggested that hormones such as estrogens and retinoids can modulate AP-1 transcriptional activity in breast cells. More recent studies suggest that ER and AP-1 interact to regulate the expression of certain estrogen and/or tamoxifen regulated genes (17). AP-1 complexes may be involved in regulating transcription of the ER gene as well (18).

These results suggest that the AP-1 complex may be involved in controlling proliferation of human breast cells; however, definitive studies demonstrating that AP-1 is critical for either breast epithelial cell proliferation or transformation have not been performed.

To address these questions we are using the extensively studied 184 series of normal human mammary epithelial cells (HMECs) isolated and characterized by Dr. Martha Stampfer (19). These cells were originally isolated from reduction mammoplasties of patients and have a normal karyotype, EGF receptors, and specific cytokeratins, suggesting that they are derived from the basal epithelial cells of the normal breast. These HMECs are primary cells which will senesce after 15-20 passages. However, by exposing these primary HMECs to the carcinogen benzo(a)pyrene, Stampfer *et al.* (19) have established multiple immortalized lines of HMECs (the 184A4N1 and 184B5 lines, and others). We are studying these carcinogen immortalized cells as well as the spontaneously immortalized HMEC line, MCF10A, derived from breast tissue obtained from a patient with multiple fibrocystic nodules (20). This cell line expresses cytokeratins and epithelial mucins consistent with a breast epithelial origin, and has cytologic characteristics of breast luminal ductal cells (21). None of the immortal cells are fully transformed; as they are not able to grow in an anchorage-independent fashion, or form tumors in nude mice. Recent reports have demonstrated that these immortalized human mammary epithelial cells can be transformed by specific oncogenes such as activated *ras* (22, 23) or *erbB2* genes (24), or by overexpression of *c-myc* or *SV40 T* genes (16). In particular, MCF10A cells can be transformed by an activated *ras* gene (23), while 184B5 cells can be fully transformed by activated *ras* genes (22), or by overexpression of c-*erbB2* (24). Many of these oncogenes are known to activate AP-1 in fibroblasts, though whether these oncogenes also activate AP-1 in breast epithelial cells is not yet known. If AP-1 is involved in regulating these processes, it might therefore serve as a target for the prevention or treatment of breast cancer. To determine the role of AP-1 in controlling breast cell growth and transformation, we proposed to test the following hypotheses:

1. Human breast epithelial cells at different stages in the carcinogenesis pathway express different levels of the AP-1 transcription factor.
2. Breast epithelial cells at these different stages have different requirements for AP-1 for their growth.
3. AP-1 transcription factor activity is necessary for *in vitro* transformation of human breast epithelial cells.

During the first year of this grant cycle we have determined the expression levels of the AP-1 family members cJun and cFos in normal, immortal, oncogene-transformed, mammary epithelial cells as well as breast cancer cells. We demonstrated that expression of AP-1 family members correlated with AP-1 DNA binding and AP-1 transcriptional activity. These studies demonstrated that AP-1 transcriptional activity decreases as mammary epithelial cells progress through the carcinogenesis pathway (normal->immortal->oncogene-transformed and breast cancer). We have also begun to investigate whether inhibition of AP-1 transcriptional activity with a cJun dominant negative inhibitor affects the growth of normal and malignant breast cells. Using two different proliferation assays we observed that normal HMECs are most sensitive to AP-1 transcriptional inhibition while breast cancer cells are less sensitive to AP-1 blockade. These studies suggest that the high level of AP-1 transcriptional activity in normal breast cells is necessary for proliferation. In the future years of this project we will determine whether mitogenesis induced by specific growth factors regulates AP-1 in normal, immortal, and cancer cells and whether AP-1 inhibitors blocks oncogene-induced transformation of HMECs.

BODY

Experimental Methods and Procedures

Primary Cell Cultures and Cell Lines

Human mammary epithelial cells and cell lines used in these studies include normal HMECs isolated from epithelial organoids of human breast from Clonetics (passages 9-10); normal 184 cells (15); 184A1 and 184B5, nontumorigenic immortal cell lines derived from benzo(a)pyrene-treated 184 cells (19); MCF10A (from Dr. J. Russo), a nontumorigenic spontaneously immortalized HMEC cell line; MCF10AneoT (from Dr. J. Russo, Fox Chase Cancer Center, Philadelphia, PA), a transformed cell line derived from MCF10A transfected by c-Ha-ras ; MCF7 WT (wild-type), a human breast adenocarcinoma cell line; MCF7 Adria, a doxorubicin (Adriamycin)-resistant subclone of MCF7 WT (from Dr. K. Cowan, National Cancer Institute, Bethesda, MD). Cells were grown in the following culture media: MEGM (Clonetics, San Diego, CA) for normal HMECs 184, 184A1, and 184B5 (19,25) DME/F-12 with 5% horse serum and supplements as described (20, 23) for MCF10A and MCF10AneoT [with 400 µg/ml Geneticin (G418), Life Technologies, Inc., Gaithersburg, MD]. and Improved MEM (high zinc option; Life Technologies, Inc.) supplemented with 10% FCS and penicillin/streptomycin for MCF7 WT, MCF7 Adria.

Growth Factor, Inhibitor and Antibodies:

For growth factor studies, HMEC were grown in minimal media (without growth factor/serum supplements) for at least 15 hours then stimulated using human recombinant epidermal growth factor (UBI, Lake Placid, NY) at 10 ng/ml at varying times.

Transient and Stable Transfections of breast cells:

Breast cells were transfected either by the calcium-phosphate precipitation procedure (breast cancer cells) or by Lipofectamine (Gibco/BRL, normal and immortal HMECs), as previously described (27). For stable transfections, cells were co-transfected with either pCOc-*erb*B2A (from T. Yamamoto) activated c-Ha-*ras* (R. Weinberg) and pSV2neo. G418-resistant colonies were picked after 2-4 weeks.

Measurement of AP-1 DNA Binding Activity :

Nuclear extracts were prepared as previously described (27). Briefly, cells were plated in 100 mm culture dishes and grown overnight. The media was not changed for 48 hours for continuously growing cells. For EGF induction, the cells were grown in minimal media for at least 15 hours, then EGF was added. The cells were then harvested at various times, washed with PBS, and lysed in cell lysis buffer (with NP-40). Intact nuclei were isolated by high speed centrifugation, and resuspended in nuclear suspension buffer. The nuclei were then lysed by freeze/thawing, and the protein concentration in the resulting nuclear lysate was determined by a colorimetric assay (Bio-Rad).

The DNA binding assay was performed using the Gel Shift kit (Stratagene, San Diego, CA) with 5 mg of nuclear protein as previously described (27). The gels were exposed to X-ray film and analyzed using the PhosphorImager (Molecular Dynamics, Sunnyvale, CA) to quantitate the intensity of retarded bands.

Western Analysis:

Equal amounts of protein from nuclear fractions were electrophoresed on a 12% acrylamide denaturing gel and transferred by electroblotting onto a nitrocellulose membrane (Bio-Rad). Primary antibodies used were [rabbit anti-cJun Ab-1 from Oncogene Science (Cambridge, MA), rabbit anti-cFos Ab SC-52 (Santa Cruz Biotechnology, Santa Cruz, CA), or rabbit anti-human activated c-*erb*B2 (#06-229,

UBI)] at a 1:250 dilution. Blots were developed using the enhanced chemiluminescence (ECL) procedure (Amersham).

CAT and Luciferase assays to Measure AP-1 Activity:

AP-1 transcriptional activating activity in cells was measured using either a Chloramphenicol Acetyl Transferase (CAT) assay or a Luciferase assay as previously described (27). The cells were transfected using calcium phosphate- or lipofectamine- (Gibco/BRL) mediated transfection with a reporter gene containing the CAT gene linked to a promoter which contains an AP-1 binding site (TGAG/CTCA). The CAT reporter DNA was co-transfected with 5 mg of CMV-βGal DNA (Clontech, Palo Alto, CA) to normal for transfection efficiency. Both the CAT (from Dr. M. Karin) and Luciferase reporter plasmids used were: -73/+63 ColCAT, -73/+63 ColLuc which contains a portion of the human collagenase gene promoter with a single AP-1 site; -60/+63 ColCAT, -60/+63 ColLuc containing a portion of the human collagenase gene promoter without the AP-1 site (negative control). The 1X-TRE-tk-CAT reporter contains a single copy of a synthetic AP-1 site; 5X-TRE-tk-CAT contains 5 copies of a synthetic AP-1 site; and ΔtkCAT contains the thymidine kinase promoter without an AP-1 site.

Cell Growth Assays:

Colony Formation Assay

2 X 10^5 cells were co-transfected in 35 mm wells with 0.5 ug of pCMV-ß-gal, and 0.5 ug pZeoSV (Invitrogen), and either pCMV (empty vector) or pCMV-TAM-67. Twelve hours after transfection the cells from each 35 mm well were split into four 35 mm wells. 24 hours after replating one well of cells were harvested and used to measure ß-galactosidase activity to determine transfection efficiency. Zeocin (Invitrogen) was added (final concentration of 400 ug/ml) to the three remaining wells After two weeks of selection in Zeocin, resistant colonies were stained with crystal violet and counted. Colony counts were normalized for transfection efficiency using the ß-galactosidase activity from each transfection.

Single cell proliferation assay:

Cells were co-transfected as described for the colony forming efficiency assay with 0.5 ug of pCMV-ß-gal and 5 ug of either pCMV (empty vector) or 5 ug of pCMV-TAM-67. Twelve hours after transfection the cells were trypsinized and replated at cell densities of 0.2 to 1.0 X 10^5 in 100 mm plates. After approximately three doublings the cells were fixed and stained with X-Gal to detect cells expressing ß-galactosidase *in situ*. Colonies containing blue cells were visualized by light microscopy and scored for the number of blue cells per colony.

Results

In **Specific Aim 1** we proposed to determine whether changes in AP-1 expression or activity occur as HMECs progress through different stages of carcinogenesis. We have completed the analysis of the basal and growth factor induced levels of cJun and cFos protein, AP-1 DNA binding, and AP-1 transcriptional activity in normal and immortal, and oncogene-transformed HMECs. These analyses are presented below:

AP-1 Protein Expression, DNA binding, and Transcriptional Activity in HMEC

We first determined the levels of cJun and cFos protein expression in the different HMECs. The HMECs used in this study are described in Table 1.

Table 1: HMECs used in this study.

Cells	Name	Source	Phenotype
Normal HMECs:	NHMEC	Clonetics	Senescent, anchorage-dependent
	184	M. Stampfer	
Immortal HMECs:	184A1N5	M. Stampfer	Immortal, anchorage dependent
	184B5	M. Stampfer	
	MCF10A	A. Russo	
Transformed HMECs:	MCF10AneoT (ras)	A. Russo	Immortal, anchorage-independent
(all with activated oncogenes)	MCF10A-erbB2	PI lab	
	184B5-ras	PI lab	
	184B5-erbB2	PI lab	
Breast Cancer cell lines:	MCF7 WT	K. Cowan	Cancer cells, anchorage-independent
	MCF7 Adria	K. Cowan	and tumorigenic
	BT 474	ATCC	

For these experiments nuclear extracts were made 48 hours after the cells were plated in fresh media. Western analyses of nuclear extracts using antibodies specific for cJun and cFos (Figure 1A) demonstrated that all the cells express cJun protein at varying levels. cFos is highly expressed in all normal and immortal HMEC, however only one of three tumorigenic cell lines express detectable amounts of cFos protein during exponential growth.

We then determined the functional AP-1 DNA-binding activity in the different HMEC using the electrophoretic mobility shift assay. To measure AP-1 DNA-binding activity, equal amounts of nuclear protein from exponentially growing cells were incubated with radioactively labeled AP-1 oligonucleotides. The results are shown in Figure 1B. Normal and immortal HMEC have high basal AP-1 DNA binding activity while the oncogene-transformed cell line, MCF10AneoT, and the breast cancer cell line MCF7 WT have lower AP-1 DNA binding activity. The normal HMEC have even more AP-1 DNA binding activity than MCF7 Adria cells (data not shown) which have previously been shown to express very high AP-1 DNA binding activity compared to other breast cancer cell lines (27).

To test whether the expressed AP-1 proteins can activate transcription we performed chloramphenicol acetyl transferase (CAT) assays to measure AP-1-dependent transactivating activity. Using the -73ColCAT reporter which has a portion of the collagenase promoter containing an AP-1 site linked to the thymidine kinase promoter, we determined the basal AP-1 transactivating activities in the panel of HMEC. The results were controlled for differences in transfection efficiencies between the cell lines by co-transfecting a β-galactosidase expression vector, and normalizing for β-galactosidase activity. The results of these studies are shown in Figure 1C, and demonstrate that proliferating normal, immortal and malignantly transformed HMEC differ in the level of AP-1 activity. There was a progressive decrease in AP-1 transactivating activity with the normal HMEC having the highest basal AP-1 transactivating activity,

immortal cells having less, and oncogene-transformed cells and breast cancer cells having the lowest AP-1 activity. These data correlate with the AP-1 DNA binding data shown in Figure 2B. However, the immortal cells show high DNA binding activity while their AP-1 transactivation activity is less than that in normal HMEC. These results demonstrate that normal HMEC grown in culture have very high basal cJun and cFos expression, AP-1 activity, and AP-1 transactivating activity. As the cells become progressively transformed the level of AP-1 transcriptional activity falls.

Induction of AP-1 Expression and Activity by Epidermal Growth Factor

To compare the ability of normal, immortal, oncogene-transformed HMEC and breast cancer cell lines to activate AP-1 in response to exogenous growth factor stimulation we measured EGF-induced AP-1 DNA binding and transcriptional activity in the different breast cells. To measure EGF-induced AP-1 DNA binding activity, we prepared nuclear extracts from normal, immortal, oncogene-transformed HMEC and breast cancer cell lines which were stimulated by addition of EGF for increasing times. Figure 2A shows the results of these DNA binding experiments, and demonstrates that there is only a minimal decrease in AP-1 DNA binding activity in the normal and immortal cells grown in media without growth factor supplementation. In addition, there was minimal induction of AP-1 DNA binding activity after EGF stimulation in these non-malignant cells. These results are in contrast to the strong induction of AP-1 activity in the tumorigenic MCF7 WT. Densitometric analyses of the retarded bands for specific AP-1 binding showed a maximum 6-fold increase over the 0 timepoint for MCF7 WT and a 2-fold increase for MCF10AneoT while normal and immortal HMEC only had a 1.4-fold increase over the 0 timepoint.

AP-1 transactivating activity in normal and immortal HMEC was also not increased by EGF, as shown in Figure 2B. In these experiments the cells were grown in minimal media and then stimulated with EGF (10ng/ml) for increasing times. AP-1 activity is shown relative to the AP-1 activity in each cell line without EGF supplement (0 time). These results show that under these conditions there was no significant induction of AP-1 activity by EGF in normal and immortal cells compared to the 4-fold induction over 0 time observed in the malignantly transformed lines MCF10AneoT and MCF7 WT.

These results from Figures 1 and 2 suggest that the relatively high levels of cJun and cFos expression, AP-1 DNA-binding, and transactivating activities are not reduced in HMEC cultured in media without growth factor supplementation and also that exogenous growth factor stimulation of normal HMEC does not increase the already high AP-1 activity present in these cells.

Effects of Transfection of Activated Oncogenes on AP-1 Activity of HMEC

Overexpression of oncogenes such as *jun*, *ras*, or *neu* induces increased activation of AP-1 in several cell types (29-31). To determine if overexpression of such oncogenes can increase AP-1 activity in HMEC, we transiently transfected activated oncogenes in immortal HMEC and measured AP-1 activity in these transfected cells. As shown in Figure 3A, transient transfection of c-*jun*, activated c-*erb*B2 and c-Ha-*ras* did not significantly increase AP-1 transactivating activity in immortal HMEC even with the use of different AP-1 reporter constructs (containing single or multiple copies of the AP-1 site). Transfection with activated c-*erbB2* reduced the basal AP-1 transactivating activity in these cells. This suggests that overexpression of the activated oncogenes c-*erb*B2 and c-Ha-*ras* does not significantly increase the already high AP-1 activity present in HMEC.

To further study the effects of activated oncogenes on HMEC, we also determined AP-1 activity in HMEC (184B5 and MCF10A) which had been stably transformed with activated c-*erb*B2 and c-Ha-*ras*. Expression of the activated form of either c-*erb*B2 or c-Ha-*ras* in the stable clones was confirmed by western immunoblotting (c-*erb*B2) or by reverse transcriptase-polymerase chain reaction (RT-PCR) assay (c-Ha-*ras*) (data not shown). The stable transfectants showed anchorage-independent growth by soft agar cloning, however, they are not fully malignant since they do not form tumors in nude mice (data not shown), except MCF10AneoT which has been previously shown to be tumorigenic in irradiated nude mice (22). We measured the AP-1 transactivating activity in these oncogene-transformed cells and in the parental cells and found that the AP-1 transactivating activities in oncogene-transfected cells are the same (c-Ha-*ras* 3) or may even be lower (c-*erb*B2A9 and MCF10AneoT) than the parental cells. Thus, in HMEC that were

transiently and stably transfected with oncogenes, AP-1 transactivating activity is not increased from the high levels in the parental, immortal cells.

In **Specific Aim 2** we proposed to determine whether the growth of HMECs at these different stages is differentially affected by inhibiting AP-1 activity.

To investigate the relative requirements of different breast cells for AP-1, we inhibited AP-1 activity using a specific cJun dominant-negative mutant and then measured the growth of the different cells in the presence of the AP-1 inhibitor.

The *colony forming assay* and the *single cell proliferation assay* are transient expression assays allowing analysis of breast cell proliferation.

Effect of inhibiting AP-1 transcriptional activity on breast cell proliferation

Analysis using the colony forming assay:

We investigated whether inhibition of AP-1 transcriptional activity effects HMEC proliferation using a colony forming assay. This assay has been used extensively to demonstrate the effects of tumor suppressors and oncogenes on cell growth. As described in Experimental Methods and Procedures, breast cells were cotransfected with pCMV-ß-*gal* and pZeoSV and either pCMV vector or pCMV-TAM-67. The pZeoSV plasmid contains a Zeocin resistance gene transcribed by the CMV promoter allowing selection of transfected cells with the antibiotic Zeocin. The pCMV-ß-Gal plasmid is included to normalize for transfection efficiency differences.

Colony forming assays were performed in estrogen receptor (ER) negative cancer cells (MDAMB231, MDAMB435, and MCF-7 Adria) and in ER positive cancer cells (MCF-7 WT, and T47-D) and a non-tumorigenic immortal HMEC line (184B5). The immortal HMEC 184B5 had approximately a 50% reduction of colony formation when transfected with pCMV-TAM-67. None of the breast cancer cells showed reduced colony formation efficiency when transfected with pCMV-TAM-67 and one (T47-D) showed an increase in colony formation (Fig. 4). These results show that proliferation of breast cancer cells, which have low levels of AP-1 transcriptional activity, was not reduced by inhibition of AP-1 transcriptional activity while proliferation of the immortal cell line 184B5 was suppressed by the AP-1 inhibitor.

Analysis using the single cell proliferation assay:

To confirm the above results and to extend the analysis to normal HMECs, we used a transient growth assay of single cells previously described by Timchenko *et al.* (32) in cells transfected with TAM-67 . We used this assay to analyze normal, immortal, and breast cancer cells growth in the presence of TAM-67. The cells were co-transfected with 5 ug of the expression vector pCMV (empty vector) or pCMV-TAM-67 plus 0.5 ug of pCMV-ß-gal. After allowing recovery from the transfection the cells were plated at low cell densities and cultured to allow single cells to grow into small colonies ranging from 1-20 cells. The cells were then fixed and stained *in situ* for ß-galactosidase activity and transfected cells were identified as blue cells by light microscopy. The number of transfected cells observed per colony were scored and presented as a histogram of the percentage of colonies having 1, 2, 3, or more transfected cells per colony.

Figure 5 shows single cell proliferation data obtained for one normal HMEC strain, an immortalized HMEC strain, 184B5, and three breast cancer cell lines. Normal HMEC was observed to be very sensitive to AP-1 inhibition by TAM-67. Cells transfected with the empty pCMV vector grew into colonies having up to five transfected cells while cells transfected with pCMV-TAM-67 did not grow beyond one cell. 184B5 cells transfected with pCMV-TAM-67 grew to colony sizes of no more than 5 cells/colony compared to more than 10 cells/colony when transfected with empty vector. Figure 4 also shows that 184B5 cells transfected with vector alone had approximately 50% of the colonies with 1 cell per colony compared to 70% when transfected with pCMV-TAM-67. The distributions of transfected cells per colony did not differ between the pCMV and pCMV-TAM-67 transfected breast cancer cell populations. Table 2

shows that the normal HMEC and immortal HMEC cells had significant reductions of cells/colony while the breast cancer cells did not. For normal HMEC cells this difference in the number of cells per colony between cells transfected with vector and TAM-67 was of borderline statistical significance (p=0.07), while for immortalized cells this difference was highly statistically significant (p=0.005). These results confirm that breast cancer cells are relatively resistant to the growth inhibitory effects of TAM-67 while immortal and normal breast cells are sensitive to TAM-67's growth suppressive effects. Such results suggest that normal HMECs and immortalized HMECs are more dependent on AP-1 signaling pathways than are breast cancer cells for their growth.

Table 2: Summary of single cell proliferation assay data.

Cell line	Growth of transfected cells	P-value*
Normal HMEC(Clonetics)	pCMV-TAM-67<pCMV	0.07
184B5 Immortal	pCMV-TAM-67<pCMV	0.005
MDAMB231 Breast cancer	pCMV-TAM-67=pCMV	0.86
MCF-7 wt Breast cancer	pCMV-TAM-67=pCMV	0.65
T47-D Breast cancer	pCMV-TAM-67=pCMV	0.90

*Data compared by Wilcoxon rank sum test

Discussion

During the first year of the grant cycle we have determined the expression levels of cJun and cFos, the DNA binding activity of AP-1 complexes, and the transcriptional activity of AP-1 in normal, immortal, and oncogene-transformed HMECs. In addition we have determined whether AP-1 DNA binding and transcriptional activity can be induced by growth factors in the different cells. We then determined whether proliferation of HMECs have differential sensitivity to AP-1 inhibition using the dominant negative inhibitor, TAM-67.

These studies have demonstrated that as HMECs proceed towards a more malignant state the basal AP-1 DNA binding and transcriptional activity decreases. Normal HMECs had high basal AP-1 DNA binding and AP-1 transcriptional activity which was unaffected by growth factor treatment, immortal HMECs had intermediate AP-1 DNA binding and transcriptional activity which was moderately stimulated by growth factor treatment, and oncogene-transformed and breast cancer cells had low levels of AP-1 DNA binding and transcriptional activity which was strongly inducible with growth factor treatment. Using two transient growth assays we observed that proliferation of normal HMECs with high AP-1 activity were sensitive to inhibition of AP-1, proliferation of immortalized HMECs with intermediate levels of AP-1 activity were moderately sensitive to inhibition of AP-1, and breast cancer cells with the lowest AP-1 activity were relatively insensitive to inhibition of AP-1 transcriptional activity.

The observation that HMECs which have high levels of AP-1 transcriptional activity are sensitive to AP-1 inhibition while HMECs which have low levels of AP-1 activity are insensitive may be due to differences in the dependence of these cells on mitogenic growth factors for proliferation. Our observation that the high basal levels of AP-1 transcriptional activity in normal HMEC was not stimulated by EGF is consistent with a model that normal HMECs are constitutively stimulated to proliferate by extracellular growth factors through a signaling pathway that activates AP-1 transcriptional activity. This is consistent with the observations of Stampfer *et al.* that normal HMEC proliferation is dependent on autocrine stimulation of the EGFR by TGF-α (34). Due to this autocrine loop normal HMEC growth is not affected by serum starvation but are growth arrested when EGFR signaling is blocked (34), and, as we observed, by inhibiting AP-1 transcriptional activity. Therefore we conclude that normal HMEC proliferation is dependent on AP-1 transcription complexes that are constitutively activated by extracellular mitogenic growth factors. These signaling pathways are altered as cells progress towards a more malignant state.

The immortalized HMEC, 184B5, has lost the TGF-α autocrine loop (34) and, therefore, are not continuously stimulated by endogenous growth factors which could explain the changes in AP-1 transcriptional activity we have observed. TGF-α is not the sole mitogenic factor to which these cells respond. We have shown that the non-specific growth factor antagonist, suramin, blocks AP-1 activity and inhibits the growth of 184B5 cells in the absence of growth factors (28) suggesting multiple autocrine loops exist which affect signal transduction pathways leading to a common target, AP-1. Transformation of cells to malignancy must further deregulate these signal transduction pathways since oncogene-transformed and breast cancer cells become serum independent and become insensitive to inhibition of AP-1 transcriptional activity. The observations that reduction of basal AP-1 transcriptional activity and increase in EGF inducibility as normal HMECs progress toward a more malignant phenotype suggests that AP-1 signal transduction pathways may be an important target to interfere with breast cell carcinogenesis.

These studies have shown the important role of AP-1 transcriptional complexes in mammary epithelial cell proliferation but the mechanisms regulating AP-1 activity in these cells are not understood. High c-Fos expression in normal and immortal HMECs may be the cause of high activity in these cells. However, it is not yet clear that the high AP-1 activity is due to c-Fos, c-Jun, or other Jun/Fos family members. Other proteins that could dimerize with c-Jun or c-Fos, such as cAMP-responsive element binding protein/activating transcription factor members, may also be contributing to the high AP-1 DNA-binding activity present in normal HMECs. In addition, the lower AP-1 activity present in tumor cell lines that have relatively high AP-1 DNA-binding activity (such as MCF7 Adria or MDAMB231 cells (27)) could be due to the presence of inhibitory proteins that dimerize with AP-1. Such inhibitory proteins that can dimerize with Jun or Fos proteins include other Jun and Fos family members, such as Jun D (35), ΔFosB (36), Fra1, or Fra2 (37), as well as other Jun and Fos dimerizing partners such as cMaf (38), or the small Maf proteins that lack the Maf transactivation domain (MafK, MafF, and MafG; 39).

The modulation of AP-1 activity that occurs during transformation of HMECs, as reflected by the difference in AP-1 transactivating activity, may also be occurring in other signal transduction pathways present in these cells. By using these HMECs as a model for carcinogenesis, we may be able to understand the signal transduction pathways and transcription factors involved in the transformation process of breast epithelial cells. The characterization of these critical signal transduction pathways may lead to the identification of novel targets for the treatment or chemoprevention of breast cancer.

Ongoing Studies

We are continuing our studies of AP-1 inhibition in immortalized HMECs and breast cancer cells. During the following years of the grant we will:

1) Determine whether AP-1 blockade suppresses mitogenesis induced by specific growth factors.

To accomplish these studies we are creating cell lines that will have inducible expression of the TAM-67 protein. We are using the reverse tetracycline inducible system (33) in breast cancer cells and immortalized cells. The creation of clones with inducible expression is carried out in two steps:

1) Creation of cell lines expressing the tetracycline inducible transcription factor, rtTA.
2) Creation of clones that have inducible TAM-67 expression using the rtTA cell lines.

Cell lines will be screened, first for induction of the TAM-67 protein, then for induction of AP-1 transcription inhibition. Cells observed to significantly inhibit AP-1 transcription will be used to study the role of AP-1 in growth factor induced mitogenesis. Cells will be grown in serum free medium or medium containing EGF, IGF, heregulin, or estrogen with and without induction of TAM-67. The growth of these cells stimulated with the different growth factors will be measured and growth in the absence and presence of the AP-1 inhibitor will be compared.

2) Determine whether AP-1 blockade inhibits the transformed-phenotype of breast cancer cells and oncogene-transformed HMECs.

We have established several clones of 184B5 cells that stably express an activated erbB2 oncogene and MCF10A cells that stably express an oncogenic ras protein. All of these clones exhibit the transformed phenotype of anchorage independent growth. These transformed HMECs and breast cancer cells will be used to determine whether inhibition of AP-1 transcriptional activity effects the transformed phenotype of breast cells. TAM-67 will be expressed in these cells and then assayed for the ability to grow in soft agar.

Progress Relative to Statement of Work:

Specific Aim 1: To determine whether changes in AP-1 expression or activity occur as HMECs progress through different stages of carcinogenesis.

We have accomplished the tasks for months 1-6 "Determine the basal and growth factor-induced expression of Jun and Fos family members in HMECs" which is described in the body of this annual report and will be presented at the "Era of Hope" meeting in November, 1997. The task for months 7-12 "Determine the functional activity of AP-1 in the different stages of HMECs, has been completed. In this annual report we describe the results of determining the functionally activity of AP-1 in the different stages of HMECs.

Specific Aim 2: To determine whether growth of HMECs at the different stages is differentially affected by inhibiting AP-1 activity.

We have determined the relative sensitivity of different HMECs to the AP-1 inhibitor TAM-67 using the colony forming assay described in the statement of work for months 7-12. In addition we used the single cell proliferation assay which allowed analysis of normal HMECs as well as immortal and breast cancer cells. These results are described in this annual report and will be presented at the "Era of Hope" meeting. We are presently continuing these studies to include additional normal and immortalized HMECs. In addition, we are also screening stable HMEC transfectants for inducible expression of TAM-67 which will be analyzed as described in the statement of work for months 25-36 and months 36-48.

Specific Aim 3: To determine whether inhibition of AP-1 activity can prevent the *in vitro* transformation of immortalized HMECs.

We have established oncogene-transformed HMECs and will determine whether TAM-67 inhibition of AP-1 transcriptional activity will alter their transformed phenotype. We are creating breast cancer cell lines that have inducible TAM-67 expression. These cells will be used to determine whether inhibition of AP-1 activity affects the transformed phenotype of tumor cells.

CONCLUSIONS

During the first year of the funding period we have shown that regulation of AP-1 transcription complex activity changes as normal human breast cells proceed towards malignancy. The changes in AP-1 transcriptional activity were shown to reflect changes in the cells dependence on AP-1 for their growth. These studies have accomplished the tasks proposed in the grant for the first 12 months. In the following years of the funding we will investigate the role of AP-1 transcriptional activity in growth factor dependent proliferation and oncogene-transformation of breast cells.

These studies have demonstrated an involvement of AP-1 transcription complexes in regulating human breast cell proliferation and will help in understanding the mechanisms leading to human breast cancer. Such information will provide the foundation for future efforts to develop agents which interfere with AP-1 signaling pathways. Such agents may be useful to block breast carcinogenesis.

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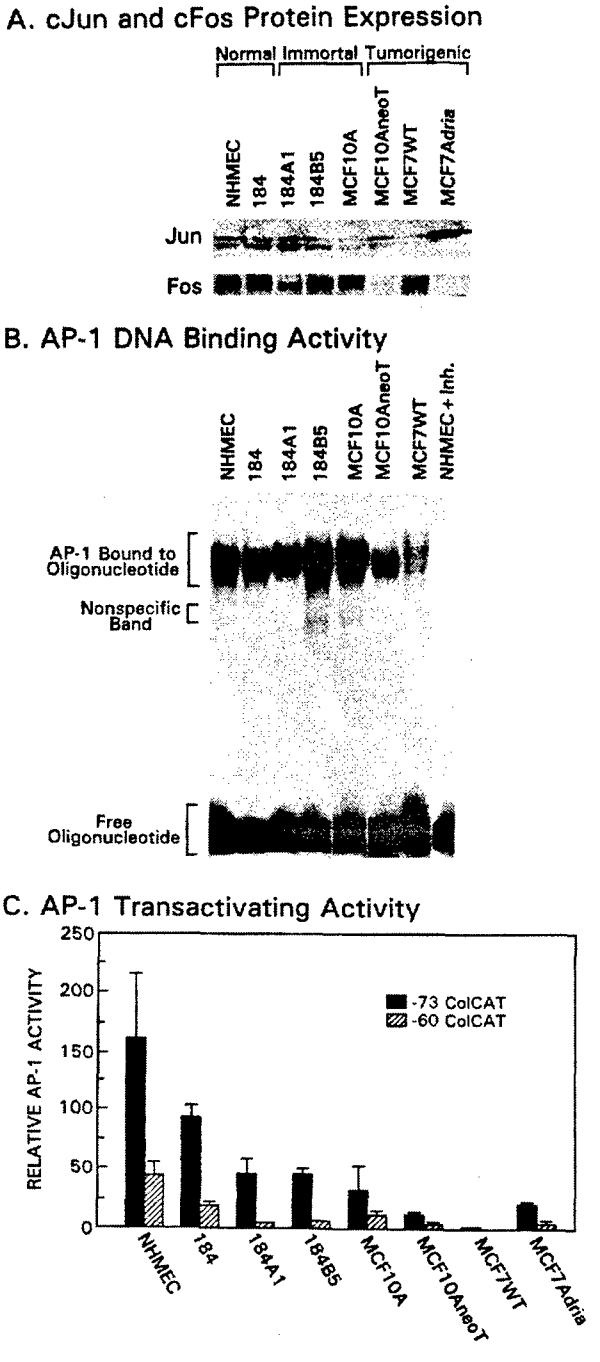
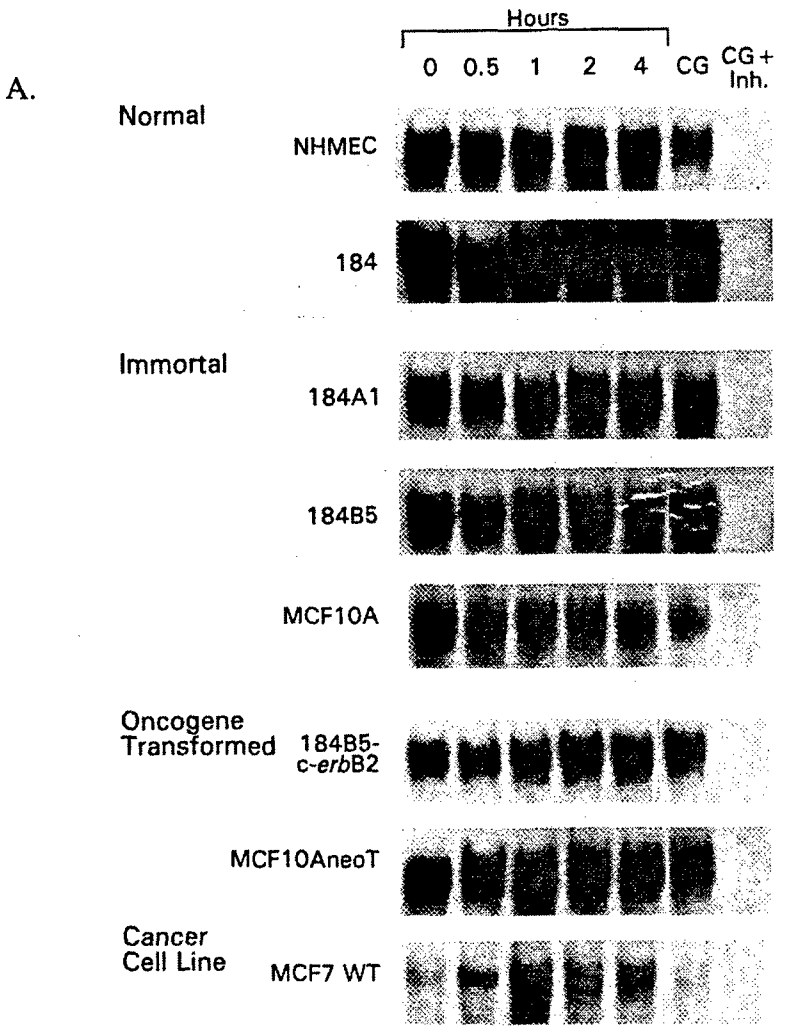


Fig. #1. AP-1 expression and activity in HMECs. A, c-Jun and c-Fos protein expression analyzed by immunoblotting. Equal amounts of nuclear extracts from continuously growing cells were detected with polyclonal anti-c-Jun Ab and anti-c-Fos Ab using the ECL detection system. B, AP-1 DNA binding activity in normal, immortal, and tumorigenic HMECs. Equal amounts of nuclear extracts from continuously growing cells were used for gel shift assays. Relative DNA-binding activity was quantitated using a Phosphoimager. *Inh.*, competitive inhibitor (unlabeled AP-1 oligonucleotide). C, AP-1 transactivating activity in normal, immortal, and tumorigenic HMECs. AP-1 activity is defined as the difference between transactivation of the -73 ColCAT reporter gene (containing a single AP-1 response element) and transactivation of the -60 ColCAT reporter gene (with the AP-1 site deleted).



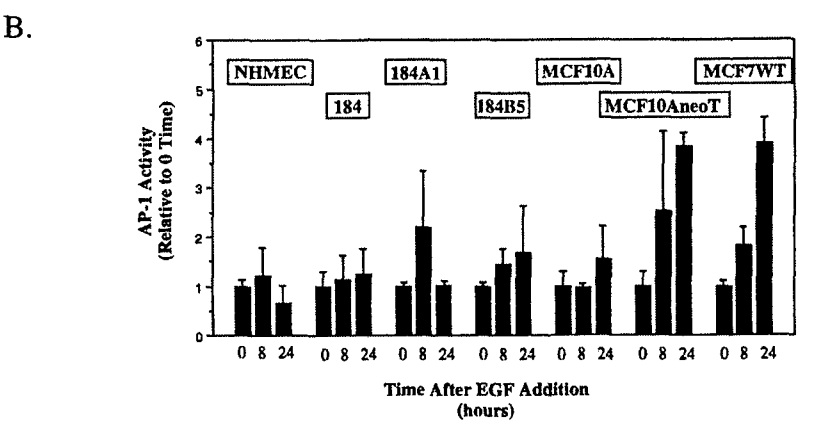


Fig. #2. Effect of EGF on AP-1 activity in HMECs. A, EGF-induced AP-1 DNA-binding activity was measured in HMECs grown in minimal medium for 15 h,followed by treatment with EGF (10 ng/ml) for the times indicated. Equal amounts of nuclear extracts were used for gel shift assays. Quantitation was done with a Phosphoimager. *CG,* continuously growing cells ; *Inh,* inhibitor (unlabeled AP-1 oligo-nucleotide). B, EGF-induced AP-1 transactivating activity was measured in HMECs grown in minimal medium for 15 h,followed by treatment with EGF (10ng/ml) for the indicated time. CAT activity was measured as described in "Experimental Methods and Procedures". Relative AP-1 activity is shown as mean fold increase of CAT activity over 0 time for each cell line studied. *Bars,* SD.

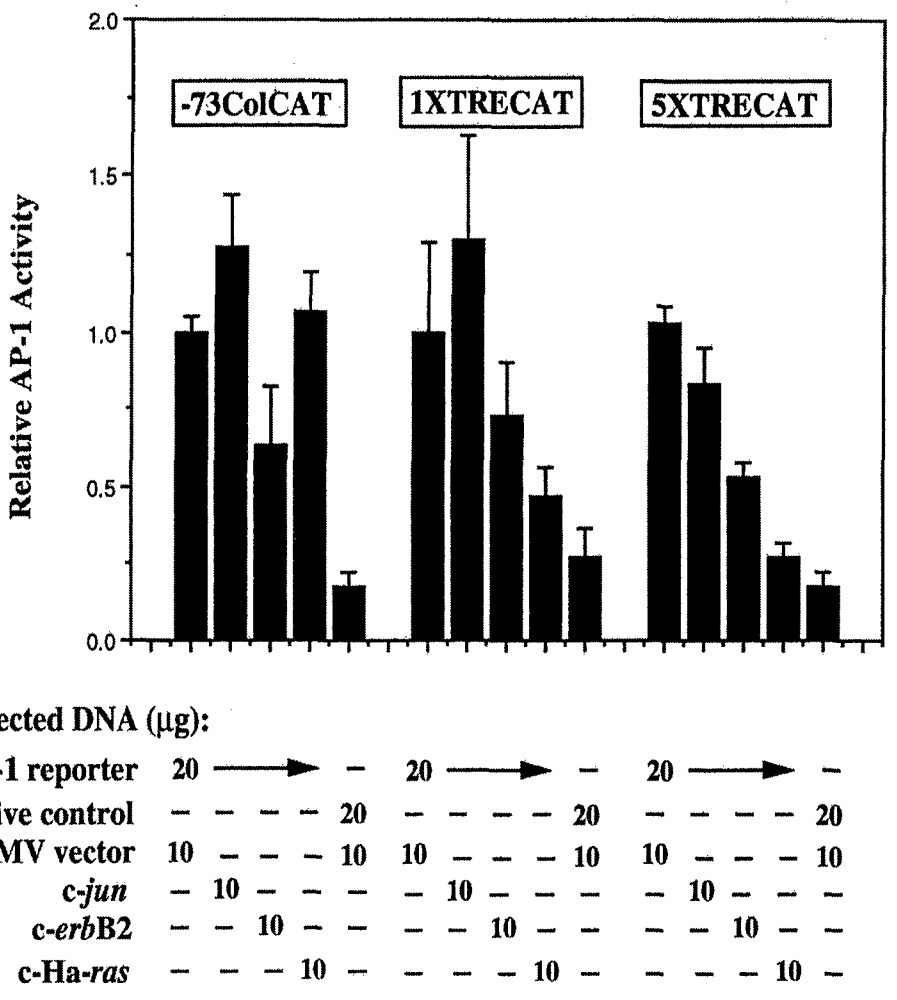


Fig. #3. AP-1 transactivting activity in 184B5 immortal cells transiently transfected with transforming oncogenes. 184B5 cells were transiently cotransfected with different AP-1 reporter plasmids [-73Col-CAT, 1X TRE (containing a single copy of a synthetic AP-1 site), and 5X TRE (containing five copies of a synthetic AP-1 site)] or their corresponding negative control CAT reporter plasmid plus CMV vector, c-*jun*, activated c-*erb*B2 or c-Ha-*ras*, and CMV- ß-Ga.l. Cells were harvested 48 h after transfection. Relative AP-1 activity is shown based on CMV vector-transfected as control. *Bars,* SD.

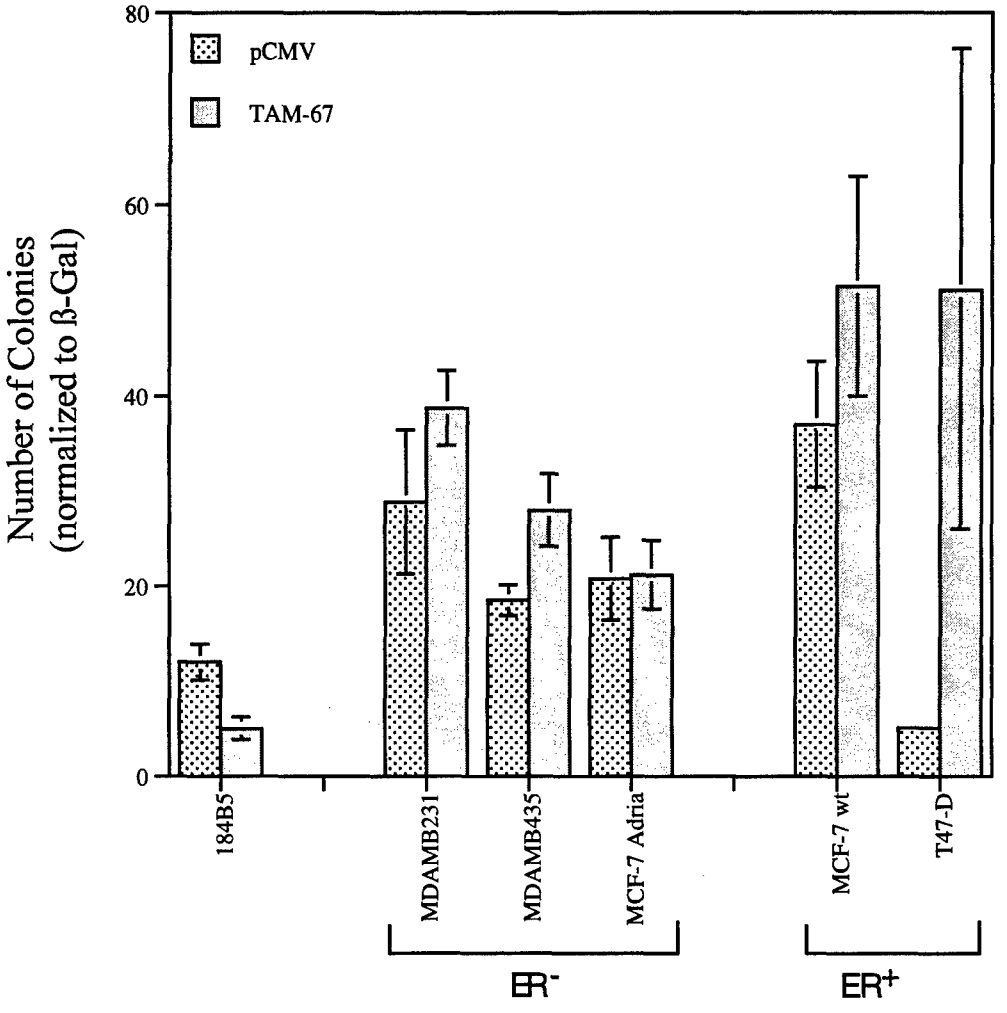


Fig. #4. Effect of TAM-67 on Colony Forming Efficiency of HMECs. The breast cancer cell lines MDAMB231, MDAMB435, MCF-7 Adria, MCF-7 wt, and T47-D, and the immortal HMEC, 184B5, were co-transfected with pZeoSV, pCMV-β-*gal*, and either pCMV or pCMV-TAM-67. Colonies were stained with crystal violet after two weeks of selection for Zeocin resistance. ER^- and ER^+ refer to estrogen receptor non-expressing and estrogen receptor expressing, respectively. All assays were done in triplicate and the number of colonies were normalized for differences in transfection efficiency using β-Gal. activity. *Bars,* indicate SEM.

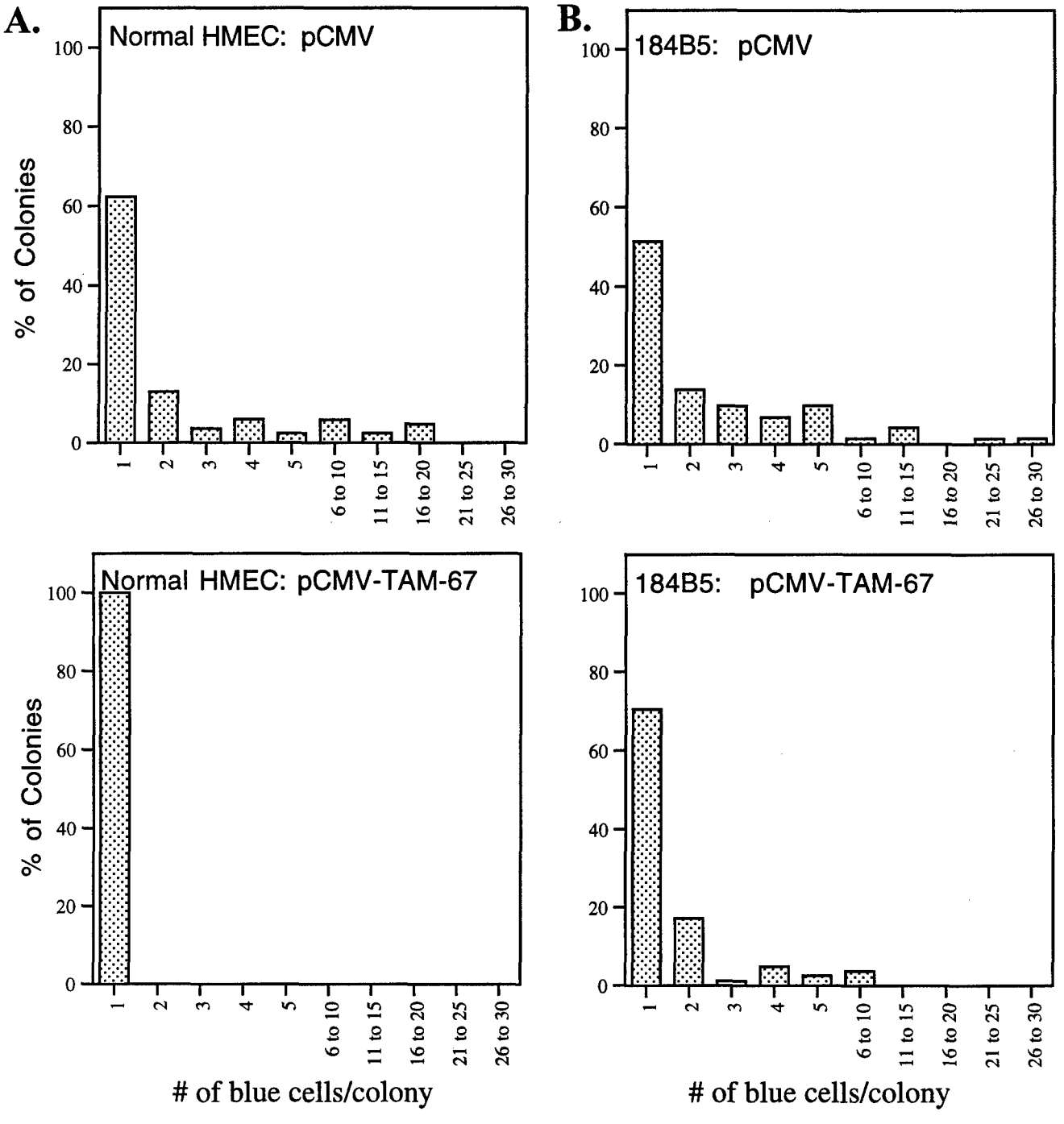
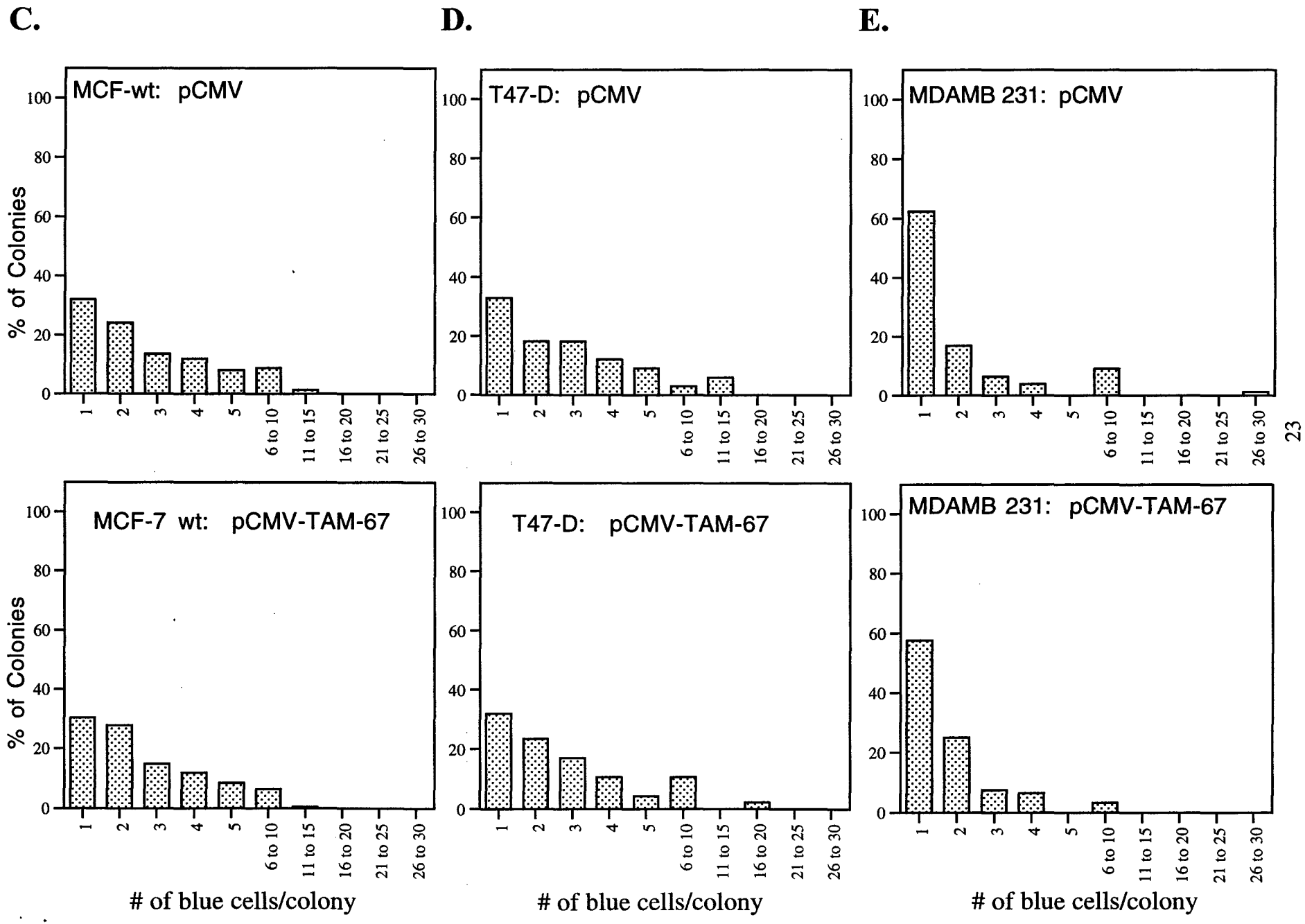


Fig. #5. Effect of expression of the AP-1 dominant negative inhibitor, TAM-67, on the proliferation of different HMECs. Normal (A), immortal (B), and tumorigenic [ER^+ (C and D), ER^- (E)] HMECs were cotransfected with pCMV-ß-gal. and either pCMV empty vector (upper panels) or pCMV-TAM-67 (lower panels). The cells were allowed to grow for approximately 3-4 doublings and then stained for *in situ* ß-Gal activity. Colonies containing blue cells were visualized by light microscopy and the number of blue cells in each colony was counted. The data is given as a histogram showing the percentage of colonies having one, or two, or three, etc, blue cells.

Figure 5 (cont'd)



APPENDICES

See attached document: Reprint of Smith *et al.* Breast cancer cells have lower activating protein 1 transcription factor activity than normal mammary epithelial cells. Cancer Research 57: 3046-3054, 1997.

[CANCER RESEARCH 57, 3046–3054, July 15, 1997]

Breast Cancer Cells Have Lower Activating Protein 1 Transcription Factor Activity than Normal Mammary Epithelial Cells[1]

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ABSTRACT

To determine whether normal breast cells have different levels of activating protein 1 (AP-1) expression and activation relative to breast cancer cells, we have compared the level of c-Jun and c-Fos expression and AP-1 activity in human mammary epithelial cells (HMECs) at different stages of transformation (normal proliferating HMECs, immortal HMECs, oncogene-transformed HMECs, and breast cancer cell lines). These studies demonstrated that normal and immortal HMECs have a high basal level of expression of cJun and cFos and higher AP-1 DNA-binding and transcriptional activating activities than do oncogene-transformed HMECs or human breast cancer cells, with a gradual decrease in AP-1 transactivating activity as cells progress through the carcinogenesis pathway (normal > immortal > oncogene-transformed > cancer cell lines). The AP-1 activity in normal or immortal cells was not modulated by growth factor supplementation or oncogene overexpression, as it is in breast cancer cells. However, the addition of suramin, a nonspecific growth factor antagonist, did inhibit AP-1 in these HMECs, suggesting that this high level of AP-1 present in normal HMECs may be due to autocrine stimulation of growth factor pathways. The differences in AP-1 activity in normal and malignant breast cells may indicate that normal cells are more dependent on AP-1-mediated signals for their growth than are breast cancer cells.

INTRODUCTION

Multiple growth factors have been identified that stimulate the proliferation or differentiation of normal HMECs[4] and breast cancer cells (reviewed in (1–3)). EGF stimulates the growth of normal HMECs (2) as well as breast cancer cells (1, 3), and heregulin has been found to modulate the growth and differentiation of immortal HMECs (4). Other hormones that affect the growth of breast cancer cells include estrogen (5, 6) and insulin-like growth factors (IGF-1 and IGF-2; Ref. 7), which induce proliferation, and retinoids, which inhibit proliferation and induce differentiation (8, 9). Steroid hormones such as estrogen and retinoids directly activate steroid hormone receptor transcription factors, whereas peptide hormones induce the activation of second messengers, which in turn activate nuclear transcription factors (10, 11). The ultimate effect of growth factor stimulation of these different signal transduction pathways is the activation of nuclear transcription factors that regulate transcription of genes. Differential expression of these target genes can determine whether the cells proliferate, differentiate, or become transformed.

The present study characterizes the expression and activity of one such transcription factor, the AP-1 complex in human breast cells, which is activated by the stimulation of mitogenic signal transduction pathways. This transcription factor is a complex of the Jun and Fos proto-oncoproteins (12, 13). AP-1 complexes are formed by dimers of Jun family members (c-Jun, JunB, and JunD) or heterodimers of the Jun family members with the Fos family members (c-Fos, Fos B, Fra-1, and Fra-2). AP-1 complexes bind to a specific target DNA site (also known as the TRE) found in the promoters of several cellular genes such as human collagenase, stromelysin, plasminogen activator (uPA), and plasminogen activator inhibitor (PAI-1) and activate the transcription of these genes (12, 13).

The AP-1 complex either positively or negatively regulates transcription of target genes, depending on the composition of the heterodimers (14–18). In response to different stimuli, such as growth factor stimulation, cellular stress, or even UV light stimulation, the expression and activity of Jun and Fos proteins are rapidly and transiently induced (19–21). In addition, tumor-promoting agents, such as TPA, have also been shown to act via the AP-1 pathway (22, 23). Several other families of transcription factors also interact with Jun and Fos proteins to affect their activity. Other leucine-zipper proteins such as members of the cAMP-responsive element binding protein/activating transcription factor family have been shown to heterodimerize with c-Jun and affect transcriptional activity of the AP-1 complex (24). Cross-coupling between c-Fos and c-Jun and the nuclear factor-κB p65 protein has also been demonstrated and was found to enhance DNA binding and transactivating activity of both nuclear factor-κB and AP-1 (25). In addition, several steroid hormone receptors, such as glucocorticoid receptor, retinoic acid receptors, and estrogen receptor, can enhance or repress AP-1 activity by a mechanism that may involve direct protein-protein interaction, a process termed transcription factor "cross-talk" (reviewed in Ref. 26).

The purpose of the present study is to determine the expression and activity of AP-1 complex in HMECs at different stages of the carcinogenesis pathway. We have shown previously that the AP-1 transcription factor is expressed in breast cancer cells and is activated by serum, TPA, or peptide growth factor stimulation in these cells (27). In this study, we determined whether AP-1 expression or activity of normal, immortal, and oncogene-transformed HMECs changes upon transformation of human breast cells. We measured the basal level and growth factor-induced expression of c-*jun* and c-*fos* genes, AP-1 DNA binding, and transactivating activities in proliferating normal, immortal, oncogene-transformed HMECs, and breast cancer cell lines. We also determined the effect of overexpressing activated oncogenes (c-*erb*B2 and c-Ha-*ras*) on the AP-1 transactivating activity in immortal nontumorigenic HMECs and in tumorigenic breast cancer cell lines. Results from these studies showed that normal HMECs have a higher basal level of AP-1 than do cancer cells, and that the high basal activity in normal and immortal HMECs is not further increased by exogenous growth factors or by the expression of activated onco-

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[4] The abbreviations used are: HMEC, human mammary epithelial cell; EGF, epidermal growth factor; IGF insulin-like growth factor; AP-1, mammalian activating protein-1; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; TRE, TPA response element; TGF, transforming growth factor; CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus; βGal, β-galactosidase; ATTC, American Type Tissue Collection.

genes. The characterization of the transcription factors activated by growth factors and oncogenes in HMECs should lead to a more complete understanding of the signal transduction pathways that control proliferation and transformation of breast cells.

MATERIALS AND METHODS

Primary Cell Cultures and Cell Lines

Human mammary epithelial cells and cell lines used in these studies include normal HMECs isolated from epithelial organoids of human breast from Clonetics (passages 9–10); normal 184 cells (2); 184A1 and 184B5, nontumorigenic immortal cell lines derived from benzo(*a*)pyrene-treated 184 cells (28); MCF10A (from Dr. J. Russo), a nontumorigenic spontaneously immortalized HMEC cell line; MCF10AneoT (from Dr. J. Russo, Fox Chase Cancer Center, Philadelphia, PA), a transformed cell line derived from MCF10A transfected by c-Ha-*ras*; MCF7 WT (wild type), a human breast adenocarcinoma cell line; MCF7 Adria, a doxorubicin (Adriamycin)-resistant subclone of MCF7 WT (from Dr. K. Cowan, National Cancer Institute, Bethesda, MD); and SKBr-3 (from American Type Culture Collection, Rockville, MD), a human breast cancer cell line with c-*erb*B2 amplification. Cells were grown in the following culture media: MEGM with or without sodium bicarbonate (Clonetics, San Diego, CA) for normal HMECs, 184, 184A1, and 184B5 (28, 29); DMEM/F-12 with 5% horse serum; and supplements as described (30, 31) for MCF10A and MCF10AneoT [with 400 μg/ml Geneticin (G418), Life Technologies, Inc., Gaithersburg, MD], and Improved MEM (high zinc option; Life Technologies, Inc.) supplemented with 10% FCS and penicillin/streptomycin for MCF7 WT, MCF7 Adria, and SKBr-3.

Growth Factor, Inhibitor, and Antibodies

For growth factor studies, HMECs were grown in minimal media (without growth factor/serum supplements) for at least 15 h and then stimulated using human recombinant EGF (UBI, Lake Placid, NY) at 10 ng/ml at varying times. Suramin was used at a concentration of 1 mg/ml. In experiments using neutralizing antibodies, cells were incubated with monoclonal antibody specific for TGF-α (R & D, Minneapolis, MN; 10 μg/ml), which has previously been shown to inhibit TGF-α-induced proliferation of the four MBr-5 epithelial cell lines (R & D, product insert) or control monoclonal antibody MOPC21 (Sigma, St. Louis, MO) and incubated for 24–48 h, then harvested for CAT assay.

Transient and Stable Transfections of HMECs

HMEC were transfected either by calcium-phosphate precipitation procedure or by Lipofectamine (Life Technologies, Inc.). Using Lipofectamine, cells plated overnight in regular media were washed twice with PBS, and 4 ml of OPTI-MEM (Life Technologies, Inc.) were added. DNA and Lipofectamine reagent (1:4 ratio) were incubated for 30 min at room temperature, added to the cells, and incubated for 6 h. The cells were washed twice with PBS and changed to regular media or serum starved. For stable transfections, cells were cotransfected with either pCOc-*erb*B2A (from T. Yamamoto, University of Tokyo, Tokyo, Japan) or T-24 activated c-Ha-*ras* (from R. Weinberg, Whitehead Institute, Cambridge, MA) and pSV2neo. Two days after transfection, the cells were harvested and grown in G418-containing media (Geneticin; Life Technologies, Inc.; 150 μg G418/ml) for selection of neomycin-resistant colonies. G418-resistant colonies were picked after 2–4 weeks.

RNA Analysis

RNA was harvested from continuously growing cells, cells grown in minimal media, or cells harvested at different time points after addition of EGF, by lysis with guanidinium isothiocyanate, and purification by centrifugation through a cesium gradient. Ten μg of total RNA were then separated using 1% agarose/formaldehyde gel electrophoresis and transferred onto Zetaprobe nylon membranes (Bio-Rad, Richmond, CA). The blots were then hybridized with ^{32}P-labeled c-*jun* or c-*fos* DNA probes, washed with high stringency (0.1× SSC, 0.1% SDS), and exposed to autoradiography.

Measurement of AP-1 DNA-binding Activity

Preparation of Nuclear Extracts. Nuclear extracts were prepared as described previously (27). Briefly, cells were plated in 100-mm culture dishes and grown overnight. The media was not changed for 48 h for continuously growing cells. For EGF induction, the cells were grown in minimal media for at least 15 h, and then EGF was added. The cells were then harvested at various times, washed with PBS, and lysed in cell lysis buffer (with NP40). Intact nuclei were isolated by high speed centrifugation and resuspended in nuclear suspension buffer. The nuclei were then lysed by freeze/thawing, and the protein concentration in the resulting nuclear lysate was determined by a colorimetric assay (Bio-Rad).

DNA Binding Assay. The DNA binding assay was performed using the Gel Shift kit (Stratagene, San Diego, CA). Briefly, 5 μg of nuclear protein were mixed with DNA binding buffer and a ^{32}P-labeled double-stranded oligonucleotide containing a single AP-1 consensus binding site (Stratagene) and incubated at room temperature for 30 min. To inhibit specific AP-1 binding, an excess of nonradiolabeled double-stranded AP-1 oligonucleotide (Promega Corp., Madison, WI) was also mixed with some control samples. Bromphenol blue dye was then added to the samples, which were then loaded onto a nondenaturing acrylamide gel to separate protein-bound DNA from free oligonucleotide. The gels were run at 4°C and then dried and exposed to X-ray film or analyzed using the PhosphorImager (Molecular Dynamics, Sunnyvale, CA) to quantitate the intensity of retarded bands.

Western Analysis

Equal amounts of protein from nuclear fractions were electrophoresed on a 12% acrylamide denaturing gel and transferred by electroblotting onto a nitrocellulose membrane (Bio-Rad). The blots were blocked using nonfat dry milk in PBS-Tween 20 (0.05%; PBS-T) for 1 h at room temperature or overnight at 4°C, washed with PBS-T, and then incubated with primary antibody [rabbit anti-c-Jun Ab-1 from Oncogene Science (Cambridge, MA), rabbit anti-cFos Ab SC-52 (Santa Cruz Biotechnology, Santa Cruz, CA), or rabbit anti-human activated c-*erb*B2 (UBI)] at a 1:250 dilution for 1 h at room temperature. Blots were washed extensively with PBS-T and then incubated with a 1:2000 dilution of horseradish peroxidase-conjugated secondary antibody (Amersham Corp.) for 1 h. Blots were washed with PBS-T and then developed using the enhanced chemiluminescence (ECL) procedure (Amersham).

CAT Assay to Measure AP-1 Activity

AP-1 transcriptional activating activity in cells was measured using a CAT assay as described previously (27). The cells were transfected using calcium phosphate-mediated or Lipofectamine (Life Technologies, Inc./BRL)-mediated transfection with a reporter gene containing the *CAT* gene linked to a promoter that contains an AP-1 binding site (TGAG/CTCA). The level of expression of this AP-1-dependent *CAT* reporter gene is defined as AP-1 transactivating activity. The CAT reporter DNA was cotransfected with 5 μg of CMV-βGal DNA (Clontech, Palo Alto, CA). The cells were harvested and lysed, and transfection efficiency was determined by measuring β-galactosidase activity in cell lysates. After normalizing for transfection efficiency, CAT activity was measured using TLC and quantitated using a PhosphorImager (Molecular Dynamics). The various CAT reporter plasmids (from Dr. M. Karin) used were: −73/+63 ColCAT, which contains a portion of the human collagenase gene promoter with a single AP-1 site; −60/+63 ColCAT, containing a portion of the human collagenase gene promoter without the AP-1 site (negative control); 1× TRECAT, containing a single copy of a synthetic AP-1 site; 5× TRECAT, containing five copies of synthetic AP-1 site; and ΔtkCAT, containing the thymidine kinase promoter without AP-1 site.

EGF Receptor Quantitation

Cells were plated in 24-well plates and grown until confluent. The medium was aspirated, and cells were repeatedly washed with HITES media (no serum; Life Technologies, Inc.). Varying concentrations of EGF and ^{125}I-labeled EGF (DuPont NEN, Boston, MA) were added sequentially and incubated at 37°C for 30 min. Excess EGF was washed off, and bound ^{125}I was counted using an LKB gamma counter. EGF receptor levels were calculated using Scatchard plot analysis.

RESULTS

Human Mammary Epithelial Cells. To determine the relative expression and activity of the AP-1 complex in normal, immortal, and malignantly transformed HMECs *in vitro,* we studied a series of cells at different stages of transformation. The following cell lines were used: normal HMEC strains (184 and NHMEC); immortal HMEC lines (184A1, 184B5, and MCF10A); oncogene-transformed HMECs (MCF10AneoT, MCF10Ac-Ha-ras3, and 184B5c-*erb*B2A9); and breast cancer cell lines (MCF7 WT MCF7 Adria and SKBr-3; Table 1). This panel of cells includes two independently isolated samples of normal mammary epithelial cells (NHMECs and 184 cells) derived from reduction mammoplasty tissue. The 184 cells have been shown previously to have a normal karyotype and to express markers typical of normal breast epithelial cells *in vivo,* including the luminal markers cytokeratin 18 and polymorphic epithelial mucins (28–30). These normal HMECs have a finite life span and do not display anchorage-independent growth properties.

We also studied three immortal HMEC lines, 184A1, 184B5, and MCF10A. The 184A1 and 184B5 cells were isolated after exposing the normal parental 184 cells to the carcinogen benzo(*a*)pyrene (28). The MCF10A cell line is an independently isolated immortal line that arose spontaneously after isolating mammary epithelial cells from an individual with fibrocystic breast disease (31, 32). These cells have specific karyotypic abnormalities and grow indefinitely in culture. However, they do not show characteristics of fully transformed malignant breast cells, because they retain growth factor dependence, do not display anchorage-independent growth, and do not form tumors in nude mice (28, 31).

The 184B5 and MCF10A immortal cell lines have been transformed with oncogenes to produce cells that will display anchorage-independent growth and tumorigenicity in nude mice. The MCF10AneoT and the MCF10Ac-Ha-ras3 cell lines are two independently transformed MCF10A clones that were produced by transfecting the MCF10A cell line with the c-Ha-*ras* oncogene (33). These cell lines show vigorous anchorage-independent growth, and the MCF10AneoT line (but not the MCF10Ac-Ha-ras3 line) has been shown to be tumorigenic in mice (33). We have also transformed the 184B5 cell line by stably transfecting activated c-*erb*B2. The resulting transformed clonogenic cell line, 184B5c-*erb*B2A9, displays anchorage-independent growth but does not form tumors in nude mice (data not shown).

We also compared the AP-1 activity of these HMECs with two breast cancer cell lines, wild-type MCF7 (Cowan strain; MCF7 WT) and an Adriamycin-resistant subclone of MCF7 WT, MCF7 Adria. We chose to use these two MCF7 cell lines to compare with normal cells, because in a previous study of AP-1 activity in multiple human breast cancer cell lines, we found that of all cell lines studied, MCF7 WT had the lowest and MCF7 Adria had the highest AP-1 activity in breast cancer cells (as defined by both AP-1 DNA-binding activity and by AP-1 transactivation activity; Ref. 27). The previous study also showed variable levels of AP-1 activity, independent of estrogen receptor level (27). Both the MCF7 WT and MCF7 Adria cell lines show anchorage-independent growth and form tumors in nude mice with estrogen supplement.

Table 1 *Cells and cell lines used in this study*

Cells	Source	Phenotype
Normal HMECs		
NHMEC	Clonetics	Senescent, anchorage-dependent
184	M. Stampfer	
Immortalized HMECs		
184A1	M. Stampfer	Immortal, anchorage-dependent
184B5	M. Stampfer	
MCF10A	J. Russo	
Oncogene-transformed HMECs		
184B5c-erbB2A9	P. Brown	Anchorage-independent, nontumorigenic
MCF10c-Ha-ras3	P. Brown	Anchorage-independent, nontumorigenic
MCF10AneoT	J. Russo	Anchorage-independent, tumorigenic
Breast cancer cell lines		
MCF7WT	K. Cowan	Anchorage-independent, tumorigenic
MCF7Adria	K. Cowan	Anchorage-independent, tumorigenic
SKBr-3	ATTC	Anchorage-independent, tumorigenic

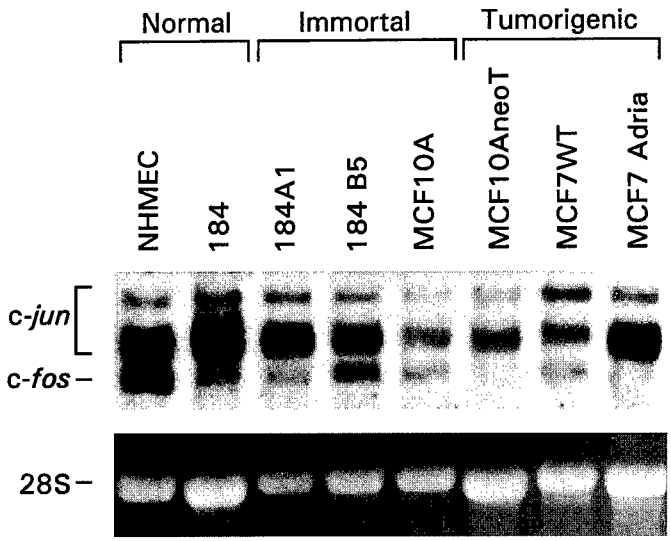

Fig. 1. c-*jun* and c-*fos* RNA expression in normal, immortal, and tumorigenic HMECs. Total RNA was harvested from continuously growing cells, and levels of c-*jun* and c-*fos* mRNA expression were analyzed by Northern blot hybridization using ^{32}P-labeled cDNA probes. The relative amount of RNA loaded in each lane is shown by ethidium bromide staining of rRNA (*28S*).

AP-1 Expression and Activity in HMECs. To study the AP-1 activity in HMECs, we measured the expression of c-*jun* and c-*fos* at the RNA, protein, and functional level in exponentially growing cells. The basal level of mRNA expression of c-*jun* and c-*fos* was determined by Northern blot analysis of RNA samples from normal, immortal, oncogene-transformed HMECs, and breast cancer cell lines that were collected during exponential growth. Although these cells grow in different media, the normal, immortal, and oncogene-transformed HMECs had similar growth rates under these conditions. The blot was hybridized to c-*jun* and c-*fos* probes, and the results are shown in Fig. 1. As observed, the cells studied express varying levels of c-*jun*. Normal HMECs have the highest c-*jun* expression, even higher than MCF7 Adria, which had been found to have the highest expression of c-*jun* of all cancer cells in a previous study (27). All normal and immortal HMECs express c-*fos* RNA, whereas in most cancer cell lines (27) and in MCF10AneoT and MCF7 Adria, c-*fos* RNA is undetected by Northern analysis. The very high level of c-*fos* RNA expressed in continuously growing normal cells is striking because c-*fos* expression is typically an immediate-early and transient response to growth factor stimulation and is often not detected by Northern analysis in exponentially growing cancer cells.

We next determined if c-*jun* and c-*fos* RNA expression correlates with the basal protein expression in exponentially growing breast cells. For these experiments, the cells were harvested 48 h after being plated in fresh media. Western analyses of nuclear extracts from these cells using antibodies specific for c-Jun and c-Fos (Fig. 2*A*) demonstrated that the basal levels of proteins in these cells correlate with RNA expression. All of the cells express c-Jun protein at varying levels. c-Fos is highly expressed in all normal and immortal HMECs; however, only one of three tumorigenic cell lines express detectable amounts of c-Fos protein during exponential growth. In general, the high level of c-Fos RNA present in the normal and immortal HMECs

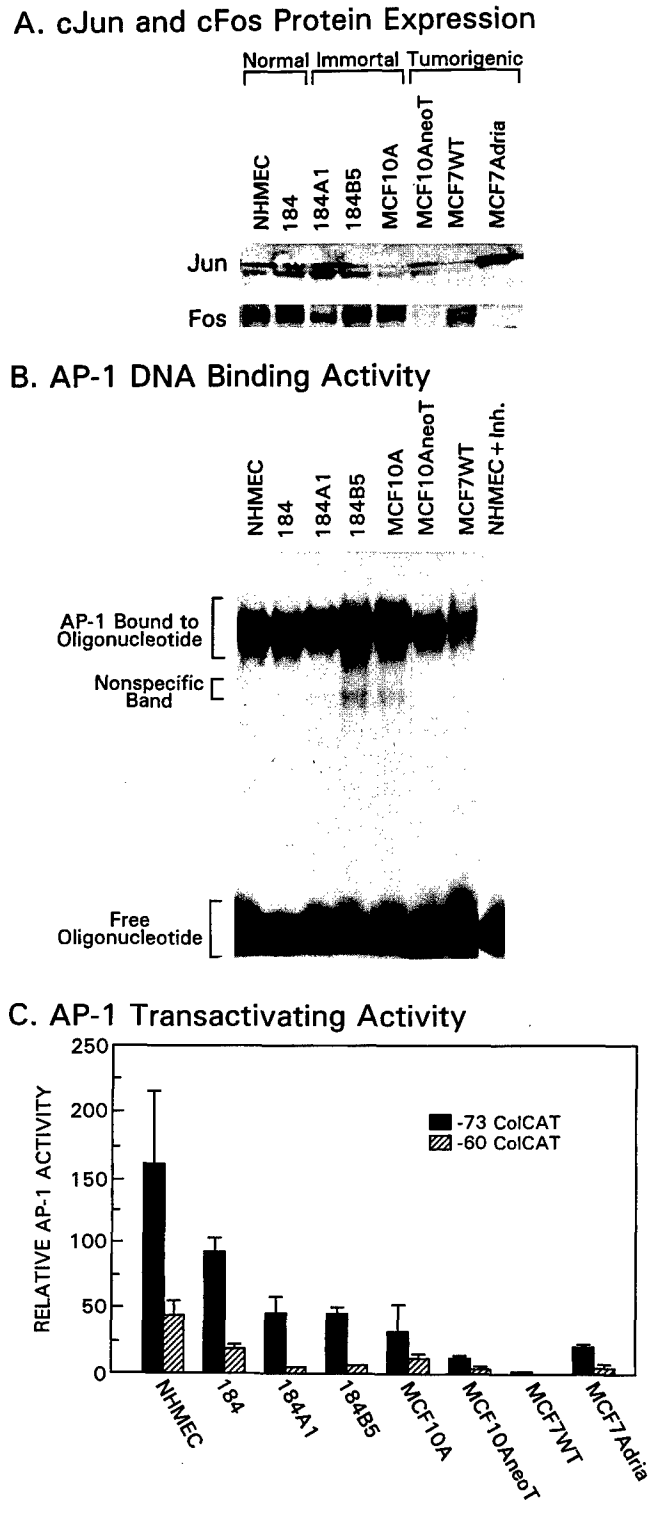

Fig. 2. AP-1 expression and activity in HMECs. *A*, c-Jun and c-Fos protein expression by Western blot analysis. Equal amounts of nuclear extracts from continuously growing cells were electrophoresed on 12% Tris-glycine gel and blotted onto nitrocellulose membrane. For detection of c-Jun and c-Fos proteins, polyclonal anti-c-Jun Ab (Ab-1) and anti-c-Fos Ab (SC-52) were used, respectively, and stained with the ECL detection kit. *B*, AP-1 DNA-binding activity in normal, immortal, and tumorigenic HMECs. DNA-binding activity was determined using a gel shift assay as described in "Materials and Methods." Continuously growing cells were harvested and used for nuclear extract preparation. Relative DNA-binding activity was quantitated using a PhosphorImager. *Inh.*, inhibitor (an unlabeled AP-1 oligonucleotide). *C*, AP-1 transactivating activity in normal, immortalized, and tumorigenic HMECs. AP-1 activity is defined as the difference between transactivation of the −73 ColCAT reporter gene (which contains a single AP-1 responsive site) and transactivation of the −60 ColCAT reporter gene (in which the AP-1 responsive site is deleted). CAT activity was measured as described in "Materials and Methods."

correlates with a high level of c-Fos protein in these cells; however the NHMEC, had higher c-Fos RNA expression than did the normal 184 cells, whereas the Western analysis shows they have similar high c-Fos protein expression. Such differences in RNA expression without major differences in c-Fos protein expression may represent differences in the translation or half-life of c-Fos in the two normal cells.

We then determined the functional AP-1 DNA-binding activity in the different HMECs using the electrophoretic mobility shift assay. To measure AP-1 DNA-binding activity, equal amounts of nuclear protein from exponentially growing cells were incubated with radioactively labeled AP-1 oligonucleotides. The results are shown in Fig. 2*B*. Normal and immortal HMECs have high basal AP-1 DNA-binding activity, whereas the oncogene-transformed cell line, MCF10AneoT, and the breast cancer cell line MCF7 WT have lower AP-1 DNA-binding activity. The normal HMECs have even more AP-1 DNA-binding activity than MCF7 Adria cells (data not shown), which have been shown previously to express very high AP-1 DNA-binding activity compared to other breast cancer cell lines (27).

To test whether the expressed AP-1 proteins can activate transcription, we performed CAT assays to measure AP-1-dependent transactivating activity. Using the −73ColCAT reporter, which has a portion of the collagenase promoter containing an AP-1 site linked to the thymidine kinase promoter, we determined the basal AP-1 transactivating activities in the panel of HMECs. The results were controlled for differences in transfection efficiencies between the cell lines by cotransfecting a βGal expression vector and normalizing for βGal activity. The results of these studies are shown in Fig. 2*C* and demonstrate that proliferating normal, immortal, and malignantly transformed HMECs differ in the level of AP-1 activity. There is a progressive decrease in AP-1 transactivating activity, with the normal HMECs having the highest basal AP-1 transactivating activity, immortal cells having less, and malignantly transformed cells having the lowest AP-1 activity. These data correlate with the AP-1 DNA-binding data shown in Fig. 2*B*. However, the immortal cells show high DNA-binding activity, whereas their AP-1 transactivity activity is less than that in normal HMECs. Such results suggest that the immortal cells express Jun and Fos DNA-binding proteins that do not activate transcription.

Induction of AP-1 Expression and Activity by EGF. To compare the ability of normal, immortal, and oncogene-transformed HMECs and breast cancer cell lines to activate AP-1 in response to exogenous growth factor stimulation, we measured EGF-induced c-*jun* and c-*fos* expression and AP-1 activity in the different breast cells. The presence of EGF receptors in these cells was first verified and quantitated by receptor binding assay using Scatchard analysis. As shown in Table 2, EGF receptor levels vary from 2.3×10^4 (MCF10AneoT) to 1×10^6

Table 2 *Quantitation of EGF receptor in normal, immortal, and tumorigenic HMECs*

Multipoint receptor binding assay using ^{125}I-labeled EGF was performed as described in "Materials and Methods."

Cells/Cell line	No. of EGF receptor molecules/cell ($\times 10^{-5}$)
Normal	
NHMEC	2.70
184	0.75
Immortalized	
184A1	2.00
184B5	2.10
MCF10A	10.00
Oncogene-transformed	
184B5c-*erb*B2	3.00
MCF10AneoT	0.23
Cancer cell lines	
MCF7 WT	0.95
MCF7 Adria	0.96

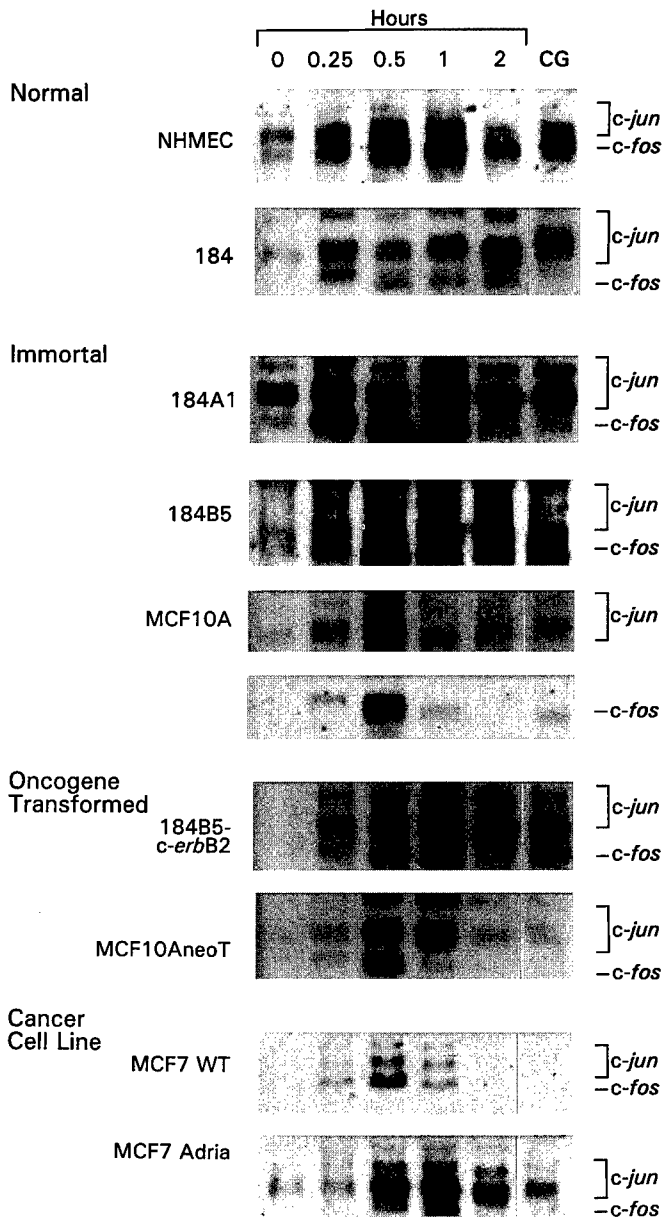

Fig. 3. Effect of EGF on c-*jun* and c-*fos* RNA expression in HMECs. The induction of c-*jun* and c-*fos* RNA by EGF was determined by Northern blot analysis using RNA isolated from HMECs grown in minimal media for 15 h, followed by treatment with EGF (10 ng/ml) for varying times. Similar amounts of RNA were loaded in each lane, as shown by ethidium bromide staining (data not shown). *CG,* continuously growing cells.

(MCF10A) EGFR molecules/cell, with the tumorigenic cell lines having fewer receptors compared to immortal cells. Normal HMECs range from 7.5×10^4 to 2.7×10^5 EGF receptors/cell. These results are comparable to previous studies of EGFR levels in HMECs (34, 35).

We next measured the c-*jun* and c-*fos* RNA expression and AP-1 activity in EGF-supplemented HMECs and breast cancer cell lines. Fig. 3 shows the results of Northern blot analysis of these EGF-supplemented cells. This figure demonstrates that EGF supplementation causes rapid and transient induction of both c-*jun* and c-*fos* RNA in all of the cells studied. These results also show that all of the HMECs can increase c-*fos* and c-*jun* expression in response to EGF at the transcriptional level.

Although EGF induces c-*jun* and c-*fos* mRNA expression in all of the breast cells, the addition of EGF failed to increase AP-1 DNA binding in normal and immortal HMECs. To measure EGF-induced AP-1 DNA-binding activity, we prepared nuclear extracts from normal, immortal, and oncogene-transformed HMECs and breast cancer cell lines that were stimulated by the addition of EGF for increasing times. Fig. 4 shows the results of these DNA-binding experiments and demonstrates that there is only a minimal decrease in AP-1 DNA-binding activity in the normal and immortal cells grown in media without growth factor supplementation. In addition, there was minimal induction of AP-1 DNA-binding activity after EGF stimulation in these nonmalignant cells. These results are in contrast to the strong induction of AP-1 activity in the tumorigenic MCF7 WT. Densitometric analyses of the retarded bands for specific AP-1 binding showed a maximum 6-fold increase over the 0 time point for MCF7 WT and a 2-fold increase for MCF10AneoT, whereas normal and immortal HMECs only had a 1.4-fold increase over the 0 time point. Western blot analysis showed that c-Jun and c-Fos proteins were present in the nuclear extracts, even after 24 h in minimal media (without growth factor supplementation), which may account for the high AP-1 DNA-binding activity observed in these cells (data not shown). It is also likely that autocrine growth factors are secreted by the HMECs under minimal media conditions, which induce high AP-1

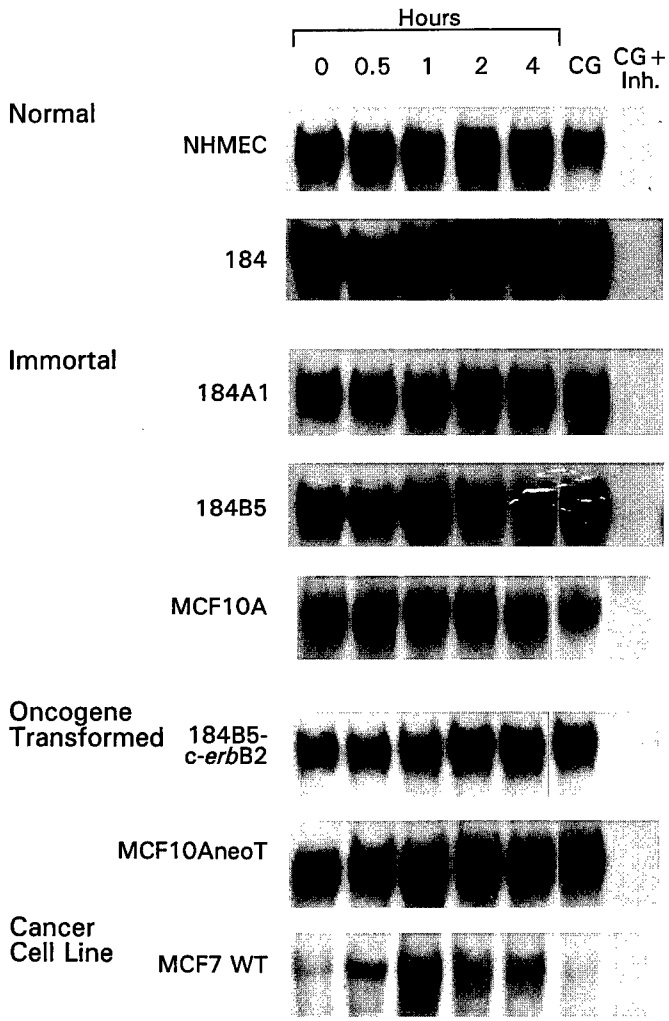

Fig. 4. Effect of EGF on AP-1 DNA-binding activity in HMECs. EGF-induced AP-1 DNA-binding activity was measured in HMECs grown in minimal media for 15 h, followed by treatment with EGF (10 ng/ml) for varying times. The cells were then harvested, and DNA-binding assay was performed as described in "Materials and Methods." Relative DNA-binding activity was quantitated using a PhosphorImager/densitometer. *CG,* continuously growing cells; *Inh.,* inhibitor (an unlabeled AP-1 oligonucleotide).

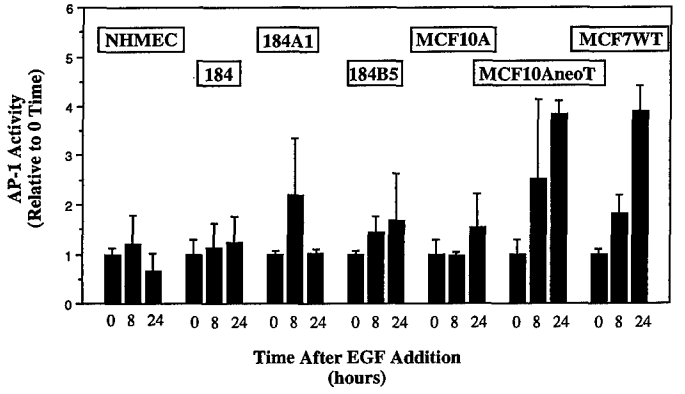

Fig. 5. Effect of EGF on AP-1 transactivating activity in HMECs. EGF-induced AP-1 transactivating activity was measured in HMECs grown in minimal media for 15 h, followed by treatment with EGF (10 ng/ml) and harvested at 0, 8, and 24 h. CAT activity was measured as described in "Materials and Methods." Relative AP-1 activity is shown as mean fold increase of CAT activity over 0 time for each cell line studied. *Bars,* SD.

activity. HMECs have previously been shown to have such autocrine loops involving the EGF receptor (34–36).

AP-1 transactivating activity in normal and immortal HMECs was also not increased by EGF, as shown in Fig. 5. In these experiments, the cells were grown in minimal media and then stimulated with EGF (10 ng/ml) for increasing times. AP-1 activity is shown relative to the AP-1 activity in each cell line without EGF supplement (0 time). These results show that under these conditions, there was no significant induction of AP-1 activity by EGF in normal and immortal cells compared to the 4-fold induction over 0 time observed in the malignantly transformed lines MCF10AneoT and MCF7 WT.

These results from Figs. 4 and 5 suggest that the relatively high levels of c-Jun and c-Fos expression, AP-1 DNA-binding, and transactivating activities are not reduced in HMECs cultured in media without growth factor supplementation and also that exogenous growth factor stimulation of normal HMECs does not increase the already high AP-1 activity present in these cells.

Inhibition of AP-1 Activity in Immortal HMECs. Because normal HMECs have been shown to produce endogenous TGF-α (34–36), it is possible that the AP-1 complex is constantly being activated by an autocrine growth factor loop in the absence of exogenous growth factors. Such autocrine stimulation could account for the high AP-1 DNA-binding and transactivating activities observed in normal and immortal HMECs, even when they are deprived of exogenous growth factors. We, therefore, attempted to lower the endogenous AP-1 activity by blocking such autocrine growth factor loops. To address this issue, we first used a nonspecific growth factor antagonist, suramin, to block the basal AP-1 transactivating activity in immortal cells grown without growth factor supplements (Fig. 6). Suramin has been shown to stimulate human breast cancer cell growth at low concentrations and inhibit growth at high concentrations (37). This suramin-induced inhibition of cell proliferation can be abolished by the addition of IGF-1, basic fibroblast growth factor, or even estrogen, confirming its nonspecific action (37). As shown in Fig. 6, AP-1 transactivating activity of 184B5 cells was decreased in the presence of suramin (1 mg/ml) relative to minimal media after 24–72 h. Suramin also inhibits the growth of these HMECs (data not shown). 184B5 has been shown to secrete lower levels of TGF-α compared to the parental 184 cells (2). When neutralizing antibodies specific for TGF-α were added to 184B5 cells, AP-1 transactivating activity was not inhibited, even after 48 h (data not shown), indicating that the increased AP-1 activity is not caused solely by autocrine stimulation by TGF-α via the EGF receptor. Such results are consistent with the studies reported by Stampfer *et al.* (36) in which c-*jun* and c-*fos* RNA expression were not decreased in 184B5 cells by EGF withdrawal or EGF receptor blockade using a blocking antibody, except for a transient decrease in c-*fos* RNA expression at 24 h. The results presented here suggest that growth factors other than TGF-α may be responsible for the high AP-1 activity observed in immortal HMECs.

Effects of Transfection of Activated Oncogenes on AP-1 Activity of HMECs. In other cell types, overexpression of oncogenes such as *jun, ras,* or *neu* induces increased activation of AP-1 (38–41). To determine whether overexpression of such oncogenes can increase AP-1 activity in HMECs, we transiently transfected activated oncogenes in immortal HMECs and measured AP-1 activity in these transfected cells. As shown in Fig. 7*A,* transient transfection of c-*jun* and activated c-*erb*B2 and c-Ha-*ras* did not significantly increase AP-1 transactivating activity in immortal HMECs, even with the use of different AP-1 reporter constructs (containing single or multiple copies of the AP-1 site). Transfection with activated c-*erbB2* reduced the basal AP-1 transactivating activity in these cells. This suggests that overexpression of the activated oncogenes c-*erb*B2 and c-Ha-*ras* does not significantly increase the already high AP-1 activity present in HMECs.

Transient transfection of c-*jun* or activated c-Ha-*ras* in MCF7 WT cells induced a 2-fold increase in AP-1 transactivating activity, whereas transient transfection of c-*jun* increased AP-1 activity by 7-fold in another breast cancer cell line, SKBr-3 (Fig. 7*B*). SKBr-3 has been shown to have an intermediate level of AP-1 activity compared to MCF7 WT and MCF7 Adria (27). Transient transfection of activated c-*erb*B2 did not change the AP-1 transactivating activity in either of the cancer cell lines. These results show that AP-1 transactivating activity can be induced by overexpression of c-*jun* in breast cancer cell lines but not in immortal HMECs.

To further study the effects of activated oncogenes on HMECs, we also determined AP-1 activity in HMECs (184B5 and MCF10A) that had been stably transformed with activated c-*erb*B2 and c-Ha-*ras*. Expression of the activated form of either c-*erb*B2 or c-Ha-*ras* in the stable clones was confirmed by Western immunoblotting (c-*erb*B2) or by reverse transcription-PCR assay (c-Ha-*ras*; data not shown). The stable transfectants showed anchorage-independent growth by soft agar cloning; however, they are not fully malignant because they do not form tumors in nude mice (data not shown), except MCF10AneoT, which has been shown previously to be tumorigenic in irradiated nude mice (33). We measured the AP-1 transactivating activity in these oncogene-transformed cells and in the parental cells and found that the AP-1 transactivating activities in oncogene-transfected cells are the same (c-Ha-*ras* 3) or may even be lower (c-*erb*B2A9 and MCF10AneoT) than the parental cells (Fig. 8). Thus, in

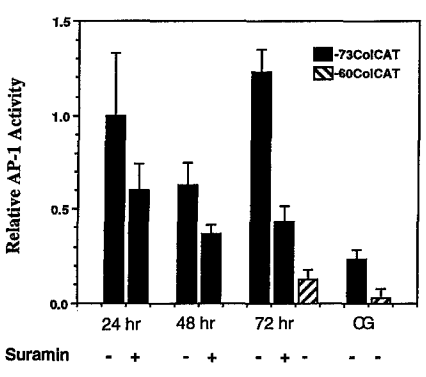

Fig. 6. AP-1 transactivating activity in 184B5 immortal cells treated with suramin. 184B5 cells were cotransfected with AP-1 reporter plasmid (−73ColCAT) and CMV-βGal, grown in minimal media for different time points with or without suramin (1 mg/ml), and harvested for CAT assay. Relative AP-1 transactivating activity is based on 24-h serum deprivation without suramin. *Bars,* SD.

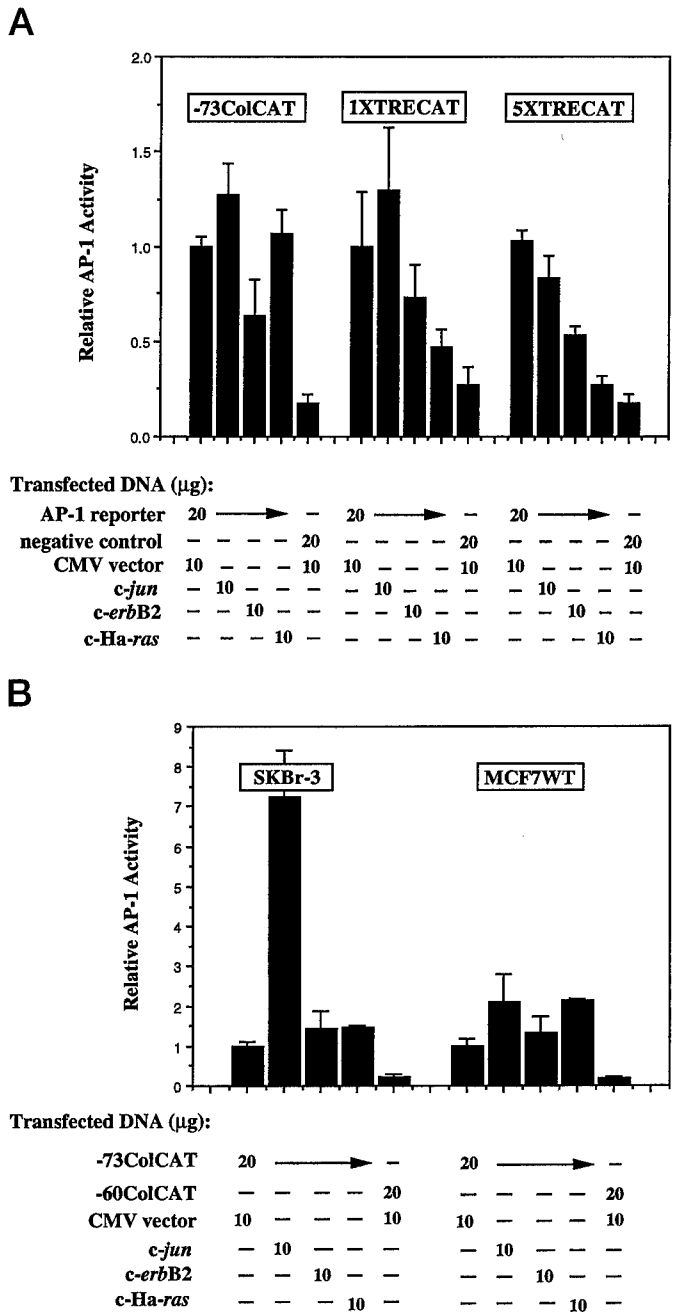

Fig. 7. *A*, AP-1 transactivating activity in 184B5 immortal cells transiently transfected with transforming oncogenes. 184B5 cells were transiently cotransfected with different AP-1 reporter plasmids [−73ColCAT, 1× TRE (containing a single copy of synthetic AP-1 site), and 5× TRE (containing five copies of synthetic AP-1 site)] or their corresponding negative control CAT reporter plasmid plus CMV vector, c-*jun*, activated c-*erb*B2 or c-Ha-*ras*, and CMV-βGal. Cells were harvested after 48 h, and CAT activity was measured as described in "Materials and Methods." Relative AP-1 activity is shown based on CMV vector-transfected as control. *Bars*, SD. *B*, AP-1 transactivating activity in human breast cancer cell lines transiently transfected with transforming oncogenes. SKBr-3 and MCF7 WT cells were transiently cotransfected with c-*jun*, activated c-*erb*B2 or c-Ha-*ras*, −73ColCAT reporter plasmid, and CMV-βGal. Cells were harvested after 48 h, and CAT activity was measured as described in "Materials and Methods." Relative AP-1 activity is shown based on CMV vector-transfected as control. *Bars*, SD.

HMECs that were transiently and stably transfected with oncogenes, AP-1 transactivating activity is not increased from the high levels in the parental, immortal cells.

DISCUSSION

To determine whether changes in AP-1 transcription factor activity occur as HMECs as progress from normal cells with finite life spans to cancer cells, we compared AP-1 expression and activity in two independently isolated normal (nonimmortalized, nontumorigenic) HMECs (NHMEC and 184), three immortal, nontumorigenic cell lines (184A1, 184B5, and MCF10A), oncogene-transformed cell lines (MCF10AneoT and 184B5c-*erb*B2A9), and two human breast adenocarcinoma cell lines derived from MCF7 cells (MCF7 WT and MCF7 Adria). On the basis of the present results, we conclude that proliferating HMECs at different stages in the carcinogenesis pathway display a progressive decrease in AP-1 activity, with normal mammary epithelial cells having the highest AP-1 transactivating activity, immortal cells having intermediate AP-1 activity, and cancer cells having the lowest AP-1 activity. In addition, normal and immortal HMECs do not increase their already high AP-1 DNA-binding and transactivating activities in response to addition of EGF, whereas cancer cells show an increase in AP-1 activity after EGF stimulation.

Oncogene overexpression, which caused malignant transformation of the HMECs, did not increase the AP-1 activity in immortal HMECs and, in some cases, decreased AP-1 transactivating activity. Transfection of activated forms of c-*erb*B2 or c-Ha-*ras* has been shown to activate AP-1 transcriptional activation in other cells, such as rodent fibroblasts. Results from this study suggest that, in HMECs, activated c-*erb*B2 and c-Ha-*ras* may instead be activating other transcription factors that are inhibitory to AP-1 transactivating activity (such as JunB or Fra-1; Refs. 14, 15, and 42).

These studies demonstrate that immortal and malignantly transformed HMECs differ in their ability to modulate AP-1 activity in response to exogenous growth factor and oncogene overexpression. These stimuli, which typically would increase AP-1 activity in fibroblasts (12, 40, 41), do not change the chronically high AP-1 activity in immortal HMECs.

The progressive decline in AP-1 transactivating activity as HMECs become transformed is in contrast to the changes in AP-1 seen in studies of keratinocyte transformation. In previous studies, Dong *et al.* (43) demonstrated that AP-1 activity in keratinocytes increases as they are transformed by TPA or EGF stimulation, and that blockade of AP-1 prevents transformation. In another study of skin carcinogenesis, Domann *et al.* (44) observed a similar increase in AP-1 activity when mouse epidermal cells were transformed by X-irradiation. The results from these studies in keratinocytes and the present studies in human breast cells point out that common transcription factors such as AP-1 can be activated by different signaling pathways in different cells. Such results stress the importance of studying transformation in appropriate tissue-specific models because transformation likely occurs via distinct molecular pathways in different cell types.

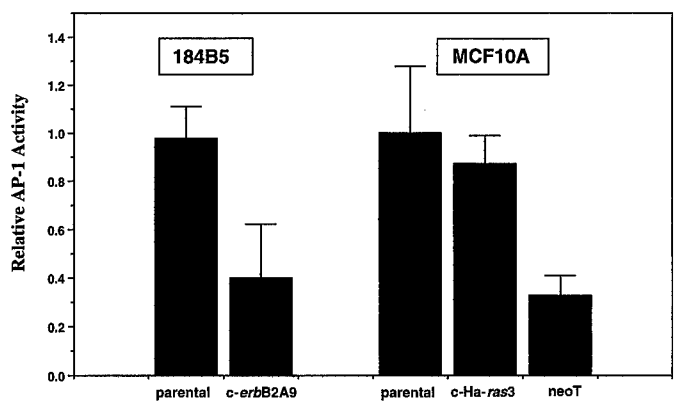

Fig. 8. AP-1 transactivating activity in oncogene-transformed stable clones. Continuously growing cells were cotransfected with −73 ColCAT reporter plasmid and CMV-βGal. CAT activity was measured, and AP-1 activity is shown relative to parental cells. *Bars*, SD.

Some possible explanations for the observed difference in the levels of AP-1 in normal and malignant HMECs include the following:

(*a*) Normal cells secrete autocrine growth factors that increase their basal AP-1 activity. The chronically high AP-1 transactivating activity in normal and immortal HMECs may be due to autocrine stimulation by growth factors as reported by other studies (35, 36). Malignantly transformed HMECs such as MCF10AneoT have lost this autocrine loop (33) and, therefore, are not continuously stimulated by endogenous growth factors. The 184B5 cells, which do not use the TGF-α autocrine loop, may be using other growth factors that activate AP-1. In our study, the use of suramin, a nonspecific growth factor antagonist, effectively inhibited the high AP-1 transactivating activity in immortal HMECs grown without growth factor supplements. This finding suggests that autocrine growth factors, possibly other than TGF-α, are chronically activating signal transduction pathways leading to higher AP-1 activation.

(*b*) Normal and immortal HMECs may be more sensitive than breast cancer cells to decreases in the level of AP-1 expression and activity. Normal and immortal HMECs may require a higher level of AP-1 activity than cancer cells to maintain growth and proliferation. Such a requirement may render nonmalignant HMECs more sensitive to fluctuations of the level of AP-1 activity. The possible requirement of immortal HMECs for high AP-1 activity to maintain their growth is supported by preliminary results from our laboratory that suggest that immortal HMECs are more sensitive to the growth-inhibitory effects of a c-*jun* dominant-negative mutant than are breast cancer cells.

(*c*) Breast cancer cells may use other signal transduction pathways. As normal cells acquire the genetic changes that lead to transformation, they may become less dependent on AP-1-mediated signal transduction pathways for continued growth. This may be reflected in the lower AP-1 expression and activity observed in oncogene-transformed and tumorigenic breast epithelial cells. Nonmalignant HMECs may require the AP-1 pathway for growth and proliferation, whereas breast cancer cells may use other signal transduction pathways to support their growth.

(*d*) High AP-1 activity in breast cancer cells may be inhibitory to the their growth. TPA, which is a potent activator of AP-1, has been shown to inhibit the growth of breast cancer cells (45, 46). In addition, overexpression of c-*jun* and c-*fos* in melanoma and lung cancer cell lines resulted in markedly reduced tumorigenicity and metastatic potential of these cells (47). These data support the hypothesis that the growth of cancer cells may be impaired by persistently high levels of AP-1 activity. Such inhibition of growth may be due to the induction of apoptosis because recent studies have also suggested a critical role of c-Jun in apoptosis in cancer cells (48, 49). Overexpression of a dominant-negative mutant of c-Jun blocked apoptosis induced by stress or ceramide in leukemic cells (48) or by nerve growth factor deprivation in pheochromocytoma cells (49). The mechanism by which c-Jun mediates apoptosis in cancer cells is still unknown, but activated c-Jun may induce the expression of proteins required for the apoptotic response in malignant cells. Because AP-1 complex is formed by dimers of Jun and Fos family members, the expression of c-Jun or c-Fos alone is not a final indication of the level of AP-1 activity. High c-Fos expression in normal and immortal HMECs may be the cause of high AP-1 activity in these cells. However, it is not yet clear that the high AP-1 activity is due to c-Fos, c-Jun, or other Jun/Fos family members. Studies are now ongoing to determine which Jun/Fos proteins are most critical for the high AP-1 activity in normal breast cells. Other proteins that could dimerize with c-Jun or c-Fos, such as cAMP-responsive element binding protein/activating transcription factor members, may also be contributing to the high AP-1 DNA-binding activity present in normal HMECs. In addition, the lower AP-1 activity present in tumor cell lines that have relatively high AP-1 DNA-binding activity (such as MCF7Adria or MDA MB231 cells [see Chen *et al.* (27)] could be due to the presence of inhibitory proteins that dimerize with AP-1. Such inhibitory proteins that can dimerize with Jun or Fos proteins include other Jun and Fos family members [Jun D (18), ΔFosB (17), and Fra1 or Fra2 (42)] as well as other Jun and Fos dimerizing partners such as cMaf (50), or the small Maf proteins that lack the Maf transactivation domain (MafK, MafF, and MafG; Ref. 51).

Although the present report characterizes the differences in expression and activity of AP-1 transcription factor complex in HMECs *in vitro,* it is possible that AP-1 expression and activity in HMECs *in vivo* is different from that seen *in vitro*. For the present experiments, proliferating normal HMECs were used. Because these cells have a finite life span and retain many of the characteristics of normal breast epithelial cells, we consider them to be the best example of normal breast epithelial cells presently available. However, normal, nonproliferating breast cells *in vivo* may not show such high AP-1 activity. To determine the expression and activity of Jun and Fos proteins *in vivo,* it will be necessary to study their expression and functional activity in normal and malignant breast tissue specimens.

The modulation of AP-1 activity that occurs during transformation of HMECs, as reflected by the difference in AP-1 transactivating activity, may also be occurring in other signal transduction pathways present in these cells. By using these HMECs as a model for carcinogenesis, we may be able to understand the signal transduction pathways and transcription factors involved in the transformation process of breast epithelial cells. The characterization of these critical signal transduction pathways may lead to the identification of novel targets for the treatment or chemoprevention of breast cancer.

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